

Hindsight on British research foresight

The first phase of the great British foresight exercise has produced a great volume of introspection that should not unduly cramp the research councils and which could persuade the government to more intelligent policy.

WHERE does (or should) foresight figure in the planning of research? Two years ago, the British government adopted as a principle the notion that the £1 billion or more a year that it spends on civil science should be informed by some attempt to define the technical objectives at which the research community should aim. Committees have been labouring away ever since, and have now published their opinions. At the outset, the government had it in mind that its search for research objectives should serve as a catalyst for an iterative exchange of opinions between the committees and the research community on the Delphi model; that appears to have succeeded only patchily, perhaps because researchers are also busy people. Nevertheless, the foresight documents now published (there are 15 altogether, dealing with different fields) are interesting and (mostly) constructive. Collectively, their greatest virtue is that they have avoided the trap of being over-prescriptive; the practice of 'picking winners' is obviously as out of fashion as it should be, given its disastrous record.

Inevitably, the 15 panels have produced documents that are variable in tangibility, often through no fault of their own. Some, for example, are reviews of economic sectors with an inherent ragbag quality, "leisure and learning", for example. That panel addresses much of what it has to say to the British government rather than to what are called the "research providers", the new name for the research councils and their dependants. Among other things, it repeats the old claim that the government should "reconsider" some form of support for the British film industry. The essence of what it has to say is that "wealth creation" in these fields stems from the quality and diversity of the educational system, government activity on copyrights and the possibility that the famous information superhighway will benefit this sector both operationally and in improving the quality of those working on the provision of software for learning and entertainment alike.

Finance

The financial services panel, faced with almost as intangible a brief, nevertheless manages to specify a few tangible research projects. To be sure, it rehearses the familiar complaint that people leaving universities with degrees in information technology (called "IT") appear to have studied "computer science in disguise" takes the view that electronic banking and the like will be commonplace ten years from now and that computer-based systems will be better than

people at managing investment portfolios within the same time, and suggests that the academic community should be encouraged to bend its energies to the design of financial instruments, asset management and people's psychological reaction to the perception of financial risk. Especially in the wake of the spectacular collapse of Baring's Bank in February, but also because of scandals such as the mis-selling of private pension plans now coming to light, academics may well believe that this important sector of British industry needs more attention than it has been given. Otherwise, the panel becomes almost lyrical in its description of how the British insurance industry may be able to use genetic information for the better assessment of insurance risks.

Information technology

The panel on information technology is more prescriptive — on what the government should do. This is an interesting turnabout: not so long (a decade) ago, the government took the initiative (under the name Alvey) in supporting a programme of collaborative research and development with industry in just this field. That had dubious value. Now the foresight panel has produced proposals for a "creative media technology centre" to stiffen British contributions to the content of visual arts (from theatre to television), advertising included. It also wants the Department of Trade and Industry to organize a number of forums in which industries and others will collaborate on flat-screen displays, distributed information management and microelectronics. It has also written a brief for the government to follow in launching a superhighway initiative. Essentially, the panel is asking that the department should function as if it were Japan's Ministry of International Trade and Industry. Evidently the panel has not fully taken in the present British government's distaste for intervention in industry, the previous inclinations of the minister concerned, Mr Michael Heseltine, notwithstanding.

How helpful will these opinions be to the research councils (now six) supporting research at British universities as well as the funding councils that support higher educational institutions? Interestingly, almost all of the 15 panel reports make recommendations that fall within the remit of the Economic and Social Research Council. That is not surprising. To the extent that the panels are as much concerned with the neglect of technology that already exists as with innovation in the strict sense, it is natural that they should be concerned with the improvement of the social climate for



economic activity in Britain. (Two centuries ago, James Watt and Matthew Boulton would not have been able to build a business selling new-fangled steam engines had it not been for the existence of joint-stock companies to moderate financial risk and commercial banks to lend them money.) On the face of things, this common theme implies a bigger budget for the smallest of the research councils. Whether the Office of Science and Technology will succeed in persuading other government departments of the need for legislative and regulatory change is another matter.

The panels representing the harder sciences naturally have more specific recommendations to make. Thus the health and life sciences panel urges greater investment (by industry as well as government) in the applications of molecular biology in fields as different as the design of new drugs, the development of diagnostic techniques and gene therapy. But the panel says the time has come to carry the search for human genes into an understanding of the working of the cell and of whole organisms, it has interesting things to say about the importance of understanding the mechanism of human ageing in the delivery of health care and it asks for a better understanding of cognition not simply so as to be able to deal with psychiatric illnesses, but also for the everyday improvement of personal efficiency. The panel backs no winners and need not constrain the decisions of the research councils in the months ahead.

So has the exercise been worthwhile? Certainly, it can have done no harm. It should also be instructive for the British government that many of the foresight panels have found themselves suggesting what the government rather than the research community should do next. Let us hope that those in government pay heed. It is also something to be grateful for that none of the panel reports will necessarily constrain the way in which the research councils do business. To be sure, on matters such as the future of thermonuclear fusion research (on which the energy panel is lukewarm, as it is on windmills), the government may decide it had better listen, but will find itself carried along by the European programme in this field. And while the panel on communications applauds British competence in the field of optoelectronics, nobody seems to have spotted that Britain lags behind the United States, Japan and France in potentially important fields such as high-precision laser spectroscopy and the design of solid-state lasers.

What happens next? In principle, British research councils are now expected to take account of the foresight documents when making new awards. But the constraint will not be as drastic as many have feared. Indeed, the most likely development is that those seeking to make their projects fundable will read the reports that concern them (uniformly priced at £15.00 at HMSO) and adjust their applications accordingly. That in itself could be valuable. Foresight, as the panels have practised it, includes much interesting hindsight about the real reasons why British industry has fallen into uncompetitive ways. The panel on the construction industry, with its lament over falling research spending and productivity, could have been written in the 1950s, and probably was. □

Good manners win out

Disputes about priority have no place in the seemingly publication of research data.

ALMOST the only public recognition of research well done is the recognition that comes from published research articles in respectable journals. That is why researchers hang so eagerly on the communications they receive from editors — and why they occasionally react with a blend of anger, depression and scorn to the news that what they wish to say cannot be published in the journal of their choice. But the authorship of scientific articles is now more than a matter of pride; so long as grant-making agencies and appointments committees rely, as they do and should not, on bibliometric indices, reference to a person in the 'acknowledgements' section of a paper rather than as an author may damage his career.

An important article in this month's *Nature Genetics* describing the possible localization on chromosome 6 of a locus of inherited susceptibility to schizophrenia (S. Wang *et al. Nature. Genet.* **10**, 41–46; 1995) appears to have offended at least two groups of collaborators.

First, the principal author of the article, Dr Scott R. Diehl of the National Institute of Dental Research (NIDR) at the National Institutes of Health (NIH), began on his study at the Virginia Commonwealth University, to which he was recruited in 1988 by Professor Kenneth S. Kendler. Diehl, with the status of principal investigator, was then awarded a research grant to look for evidence of genetic predisposition to schizophrenia in an extensive set of data collected in Ireland by Kendler, working in collaboration with Irish psychiatrists (on both sides of the Irish border). Then, it appears, Diehl and Kendler fell out over ownership of the interpretation of the data, Diehl moved to NIDR and NIH (asked to adjudicate) said that the two should seek an accommodation but that, otherwise, each should be free to publish separately.

Second, Kendler, in collaboration with a new collaborator, Dr Richard E. Straub, had analysed the original and supplementary data and had also prepared a manuscript for publication; a version of this was sent to Diehl on 15 March, among other things for his approval of the inclusion of his name (third in a list of eleven), which offer was declined. Diehl could hardly have accepted, knowing that his own article was being reviewed by *Nature Genetics*. That article acknowledges that the data had been "collected in Ireland", includes Kendler and a number of Irish names among its acknowledgements but does not mention the Virginia Commonwealth University, the Health Research Board in Dublin or the Queen's University, Belfast.

Technically, nobody has behaved wrongly. Diehl has NIH approval for separate publication. So has Kendler. If NIH had followed Solomon, it would have recommended that the article and the authors' list should be chopped in half, to see how the participants reacted. But what about the proper Irish interests, where NIH's writ does not run? Personal disputes about priority should be buried in the interests of seemingly publications, especially when third parties are concerned. □

US universities propose long-term solution to dispute on overheads

Washington. Finance chiefs at six leading US research universities are developing a plan that would tie university research overhead rates to a national formula and — they hope — end the continuing war about overhead costs with successive administrations and Congresses.

The proposal — known as the Phoenix plan, after the Arizona city in which it was first conceived — is being floated as both houses of Congress prepare budget proposals in which research overhead costs may once again come under attack.

Most universities have a single research overhead rate, calculated on the basis of administrative and facilities costs added to the cost of every application for a federal grant to come from the institution. Rates vary from 30 to 60 per cent, with publicly-funded universities charging less and private ones charging more; Stanford University, for example, currently charges 58 per cent.

In discussions with university officials, congressional staff have indicated that next week's House of Representatives budget proposal will leave overhead costs alone, but the Senate resolution may support a proposal by Hank Brown (Republican, Colorado) to limit the rate to a 40 per cent maximum.

For a private university or institution with a current rate of 60 per cent, such a proposal

would immediately cut one in every eight dollars of federal research support. The House and Senate budget proposals must be brought into line and signed by President Bill Clinton before they take effect.

The Phoenix plan has been drawn up by finance vice-presidents at six universities — the University of California system, Chicago, Cornell, Georgetown Medical School, Princeton and the University of Southern California. The six are reluctant to discuss the plan until it wins broader support. But it would use a national formula to determine the overhead rate for each institution on the basis of federally-compiled indices of local wage and construction costs.

In the past, universities have opposed such a formula because they wanted overhead costs at each institution to be "real costs" of which every dollar and cent could be verified by auditors. But the steady drum-beat of complaint from Congress about different institutions charging different rates has convinced some universities that the present system is no longer tenable.

The Phoenix plan "would put a degree of certainty into the indirect cost system because it would use a national database which would be recognized and accepted by everyone," says one official familiar with the proposal, which is now circulating among

university administrators. If they respond favourably, the authors propose a detailed study into the feasibility of the change. The plan is intended as a long-term solution to a perennial problem, the official says, and will take at least a year to finalize.

The plan is described as far simpler than an earlier White House proposal on research overhead funding (see *Nature* 373, 552; 1995). Some universities are concerned about aspects of the White House plan, including its complexity and what they see as a lack of any assurance that the new system would work before the old was abolished.

In a separate development, private research institutes have moved to pre-empt the threat that their overhead payments will be singled out for attention in this year's budget process.

A document issued by the Congressional Budget Office (CBO) in February spelling out Congress's budgetary options said that the institutes claim higher overhead payments — 63 per cent on average — than do universities, and suggests that capping their payments at 90 per cent of this year's level could save \$2.2 billion over five years.

Kenneth Trevett of the Dana-Farber Cancer Institute, Boston, who is also president of the Association of Independent Research Institutes, wrote to the director of the CBO last week, saying that the institutes' overhead rates are in line with those at private universities. He asked the CBO to issue a correction to its report, adding that its suggestion "would have little effect on the deficit while dramatically penalizing one group of institutions". **Colin Macilwain**

New editor is appointed at *Nature*

Philip Campbell, the editor of *Physics World*, has been appointed Editor of *Nature* from 8 December of this year. *Physics World* is an international monthly publication of the UK Institute of Physics (IoP), whose circulation has grown from 12,000 to 25,000 during the seven years since it was established, all of them under Campbell's direction.

Campbell, 44, is an old *Nature* hand, having joined the physical sciences section in 1979 after a period of postdoctoral research in atmospheric sciences. He became physical sciences editor of *Nature* in 1981.

He joined *Physics World* sufficiently in advance of its first issue to be responsible for the editorial planning of the magazine, which was formed out of two existing IoP journals.

In recognition of his creation and development of the publication, he was last month elected a Fellow of the IoP, a

distinction awarded to those who have made outstanding contributions to the physics community. He is also a Fellow of the Royal Astronomical Society.

Campbell said earlier this week that "the greatest challenge and pleasure of being an editor is working with skilled and imaginative colleagues to make a real impact on readers, while maintaining the highest scientific standards."

He added: "*Nature* offers great scope for that, as it has shown time and time again. It also provides a superb dissemination service for the world's scientists. Taking such a publication forward when science and scientific publishing are both developing so rapidly is a truly exhilarating prospect."

Sir John Maddox, the present editor of *Nature*, will retire on 8 December, but will then be called Editor Emeritus of *Nature*. In this role he may by invitation continue to contribute to the journal. □



Campbell: exhilarated.

Briton and Dane share Crafoord prize

London. The Royal Swedish Academy of Science has decided to award the 1995 Crafoord prize for geoscience — the equivalent of the Nobel prize for fields unrecognized by the Nobel foundation — jointly to a Danish and a British scientist for their scientific achievements in the study of past climate variation.

The prize, a gold medal and a sum of 2.8 million Swedish kroner (US\$384,000), will be shared by Willi Dansgaard, professor of geophysics at the University of Copenhagen's Niels Bohr Institute, and Nicholas J. Shackleton of the University of Cambridge's subdepartment of Quaternary research. The award will be presented at a ceremony in Stockholm on 28 September by King Carl Gustav of Sweden. **David Dickson**

FBI confirms no evidence to back Oppenheimer charges

Washington. US President Bill Clinton's Foreign Intelligence Advisory Board (PFIAB) this week released a brief statement from the Federal Bureau of Investigation (FBI) saying that the bureau had found no "credible evidence" behind allegations that four senior physicists on the Manhattan Project spied for the Soviet Union, and that it believes the allegations to be false (see *Nature* 374, 581; 1995).

The allegations were made last year by former Soviet spymaster Pavel Sudoplatov in his book, *Special Tasks*. Since then, they have been furiously disputed by US physicists and friends of the four men, all of whom are now dead.

This week's statement takes the form of a letter sent to Les Aspin, chairman of PFIAB, from Louis Freeh, director of the FBI. The letter says that the bureau "is not in possession of any credible evidence that would suggest that Niels Bohr, Enrico Fermi, Robert Oppenheimer, or Leo Szilard engaged in any espionage activity on behalf of any foreign power to include that involving atomic bomb secrets."

The letter continues: "Indeed, the FBI has classified information available that

argues against the conclusions reached by the author of *Special Tasks*. The FBI, therefore, considers such allegations to be unfounded."

At a press briefing in Washington at which the statement was released, Aspin said that type of classified information referred to by the FBI included lists of names of people who had helped the Soviet Union with its nuclear weapons programme, and had been obtained by the US government. "These lists exist and these [four] names are not on them," Aspin said.

Aspin characterized the investigation as "a small effort" which had focused on a single question — whether evidence existed to confirm or refute Sudoplatov's allegations. The FBI, he said, had found no evidence either way in its files. But it had concluded on the basis of the "other evidence" that Sudoplatov was wrong.

The investigation was instigated by Sid Drell, deputy director of the Stanford Linear Accelerator Center in California and a member of PFIAB. But Aspin agreed that it may not be the last word on the matter.

At Monday's briefing, Jerrold Schecter, who helped Sudoplatov write the book, called on the US government to declassify records of coded messages which were passed from Soviet agents in the United States to Moscow at the time but were intercepted by US officials. Schecter said that these intercepts would resolve the question.

Colin Macilwain

IMAGE
UNAVAILABLE
FOR
COPYRIGHT
REASONS

Aspin: 'names not on
list of Soviet spies'.

Anomaly admitted in 'first' AIDS case

London. The researcher responsible for claims — recently called into question — that the world's first AIDS patient was a 25-year-old apprentice printer who died in Britain in 1959 has acknowledged he knew his results may have been anomalous, even at the time of conducting his analysis.

In a co-authored letter to *The Lancet*, Gerald Corbitt, director of clinical virology at the Manchester Royal Infirmary, also admits that he was aware in 1992 that the apprentice, David Carr, could not have died of AIDS, as the HIV detected in tissue samples analysed in 1990 were from "a relatively modern strain".

Corbitt was responding to findings published last month by David Ho, director of the Aaron Diamond AIDS Research Center in New York, which challenge the researcher's original claims that Carr was the first recorded AIDS death (see *Nature* 374, 503; 1995).

Having reanalysed Carr's tissue samples, Ho, who had been cooperating

with Corbitt, concluded that the strain of HIV detected could not possibly have existed in 1959. Further samples taken from Carr's kidney and bone marrow subsequently tested HIV-negative, suggesting that the collection of tissues could have come from two separate individuals.

In a letter commenting on Ho's findings and published in *The Lancet* on 22 April, Corbitt says that the findings "confirm our own view", based on "sequencing work done in the two years after publication."

Asked this week why he failed to publish his doubts about his initial findings sooner, and thus clear up the confusion over the identity of the first AIDS case, Corbitt says he felt no reason to do so.

Corbitt contends that his original 1990 letter to *The Lancet*, was not intended as "a peer-reviewed article" on the origin of AIDS. The motivation behind analysing Carr's tissue, he adds, was simply "to see whether we could use PCR on archival material".

Ehsan Masood

MRC agrees terms of access to gene sequence databank

London. Britain's Medical Research Council (MRC) has reached agreement with Smith-Kline Beecham (SB) and the Institute for Genomic Research (TIGR) on the terms under which MRC scientists will be allowed access to 150,000 human gene sequences generated by collaboration between by TIGR and Human Genome Sciences Inc.

Initially, MRC officials had voiced strong criticism of some of the terms being required by HGS for access by academic scientists to its databases, and had told scientists funded by the council, including 4000 scientists working in British universities and medical schools, not to accept the conditions being offered (see *Nature* 373, 376; 1995).

In particular, their concern had focused on proposed clauses limiting the freedom of scientists to discuss work using information from the data-base with colleagues in the same laboratory, and others giving HGS a first option on any commercially-valuable research results emerging from the use of the data.

David Owen, head of licensing for the MRC, said on Monday that the agreement that has now been negotiated with SB, one of Britain's largest pharmaceutical companies, meets many of these early concerns. "We have tried to go for a UK solution," says Owen, arguing that the way that the obligations of researchers in early draft agreements proposed by HGS was to be met was "not as clear as it could have been."

As far as the first option clause is concerned, for example, Owen says that the new terms establish that SB/TIGR "must present us with firm evidence that they are going to exploit the discovery and not sit on it."

Craig Venter, the president and director of TIGR, said that he was "very pleased" that agreement had been reached with the MRC. Venter claimed that difficulties in the past had been partly the result of confusion between TIGR and HGS. (The two bodies are formally independent, although the Human cDNA Database, which is managed and operated by TIGR, is supported financially by SB and HGS).

Last year, HGS reached agreement with the Howard Hughes Medical Institute (HHMI) on the terms under which HHMI scientists are permitted access to the data-base. According to Venter, similar agreements have now been signed with more than 50 research institutions world-wide.

However negotiations are still taking place with the National Institutes of Health (NIH), which is faced with the need to reconcile the access conditions that it is being asked to accept with the political pressures that inevitably impinge on the biomedical research agency.

David Dickson

Harvard 'starts inquiry' into UFO researcher

Boston. Harvard University is said to have set up a special faculty committee to investigate research by John Mack, professor of psychiatry at the Harvard Medical School, into the experiences of those who claim to have been abducted in Unidentified Flying Objects (UFOs).

The university has declined either to confirm or deny the existence of the committee, or to give details of its powers or the terms of reference under which it is said to have been set up.

But it is understood that the committee has been formed by Daniel Tosteson, dean of the medical school, and is headed by Arnold Relman, former editor of the *New England Journal of Medicine* and currently a professor emeritus at the medical school.

Those who say they are familiar with the investigation maintain that Mack's work with patients claiming to have been abducted by extraterrestrial beings has become a source of embarrassment to the university.

Extracts from what is purported to be a draft report of the committee criticize Mack for failing to require physical evidence from those claiming to have been abducted, and say it was "professionally irresponsible" for him to lend credence to reports of extraterrestrial contact. But supporters claim that his academic freedom is being threatened.

Mack is a former chair of the school's department of psychiatry. His bestselling book, *Abduction: Human Encounters With*

Aliens, has been promoted on television and radio talk shows throughout the country.

His critics say he has abandoned scientific objectivity and may have elicited memories of abductions through hypnosis. "Mack is an exceedingly naive man who can't believe that people lie or make up stories," says James Randi, an internationally renowned sceptic of paranormal phenomena.

Mack himself declines to discuss the activities of the committee, whose existence was reported last month in both the *Boston Globe* and the university student newspaper, the *Harvard Crimson*.

But academic UFO investigators, such as David Pritchard, a physicist at the Massachusetts Institute of Technology, believe that Harvard is infringing Mack's freedom of inquiry. "He is subject to an investigation that, as far as I can tell, has not been initiated by the faculty," says Pritchard. Other Mack sympathizers go further, calling the proceedings against him a "witch-hunt".

Beverly Rubik, director of the Center for Frontier Studies at Temple University, says that "putting the lid on Mack would be bad policy for Harvard and bad for science in general." She adds: "Science moves forward when people start asking new questions."

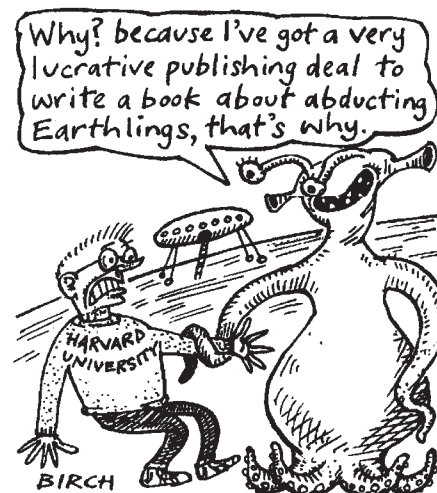
John Miller, a physician with the Southern California Permanente Medical Group, who has interviewed more than 200 individuals reporting abduction experiences, claims in a letter to the faculty committee that the

controversy "has more to do with academic freedom than [with] aliens or UFOs".

The letter was sent in response to a request by Daniel Sheehan, Mack's former lawyer based in California, who has sent similar requests to several other UFO or abduction 'experts' asking them to write statements in Mack's defence. Excerpts supposedly taken from a draft report of the committee, and quoted by Sheehan, have been transmitted over the Internet.

Roderick MacLeish, Mack's attorney, says that "Sheehan does not represent Mack, nor was he authorized to send that material over the Internet." But Pritchard claims that the tactic was successful in "raising a furor" over the Harvard inquiry and bringing the matter to public attention.

In his letter, Miller cites several weaknesses in what Sheehan claims to be the preliminary findings of the committee report. He takes issue, for example, with the com-



Canada to get own science satellite

Washington. Thanks partly to Canada's participation in the international space station, Canadian scientists will soon get their first chance in 30 years to lead the design of a scientific satellite, SciSat 1.

This is the first of two satellites that will be financed by the Canadian Space Agency (CSA) and launched by the US National Aeronautics and Space Agency in 1999 and 2004 respectively. Both will be launched without charge on a Pegasus launcher — a rocket mounted underneath an aircraft — as part of a deal to ensure continued Canadian participation in the space station.

Within the next two months, the CSA will invite proposals to design the first SciSat satellite. Gerry Atkinson, chief scientist at the agency's space sciences programme, expects about a dozen groups to apply. The winner will be chosen by early 1997.

Some scientists have suggested that the C\$35 million (US\$25 million) being made available to design and build each of the SciSats is insufficient, especially as CSA rules stipulate that instruments cannot be assembled cheaply in universities, but must be built by Canadian industry.

But Atkinson says that the sum, approved

by the Canadian government, is what the space agency had argued was needed. Canada also hopes to attract overseas partners to build one or two extra instruments — estimated to cost between C\$5 and C\$10 million each — for the first SciSat.

Fierce competition to design the first satellite is expected from three disciplines — astronomers, Earth scientists who want to study the atmosphere, and scientists interested in the interaction between solar rays and the upper atmosphere. At least one group of astronomers, led by Slavek Rucinski at the University of Toronto, is already planning a bid to use the satellite for ultraviolet astronomy.

Canada has not led a project to design and build its own scientific satellite since the 1960s, preferring to collaborate with countries such as Sweden. Atkinson concedes that this has led to "some frustration" among Canadian scientists.

The space agency considered soliciting bids for both SciSats simultaneously. But, because of the length of time before the planned launch of SciSat 2, it has decided to select only a design for the first satellite at this stage.

Colin Macilwain

mittee's insistence that Mack should have corroborated patients' stories with physical evidence of their supposed abduction. "A psychiatrist is supposed to deal with patients in a therapeutic setting, not go out in the field and gather forensic evidence," he says.

Miller also says that "at times, it may be more responsible to let patients hold onto their beliefs — even if they are based on a delusion — than to shatter those beliefs entirely". He also criticizes the fact that no members of the committee appear to have met anyone claiming to have been abducted. "I was taught at medical school that you can learn a lot from people just by listening to them carefully," says Miller.

"Although the origins of the syndrome are unknown, and uncertainties abound, one thing can be said for sure: these people, who consider themselves 'abductees,' do exist," he says. "They can be studied, and something can be learned from this process, even if the whole phenomenon turns out to be a product of the human mind."

Steve Nadis

Laser test facility 'will maintain deterrence'

Paris. The French government has approved the construction of a 1.8-megajoule laser for nuclear weapons research as part of its plans to maintain the country's nuclear deterrent without having to carry out explosive nuclear tests of its hydrogen bombs.

The laser facility is estimated to cost FF6 billion. It is almost identical to the \$1.8-billion National Ignition Facility (NIF) planned for the Lawrence Livermore National Laboratory in California (see *Nature* 371, 729: 1994).

But whereas the US decision to build NIF has provoked a vigorous debate over its impact on efforts to contain the proliferation of nuclear weapons, France's decision has been overshadowed by the question of whether the election of a new president next weekend will lead to the end of its self-imposed moratorium on explosive testing.

In France, all decisions concerning the nuclear deterrent come under the direct responsibility of the president. The moratorium was introduced in 1992 by President François Mitterrand, who, in a late conversion to the cause of non-proliferation, argued that tests could only lead to an escalation of the arms race and the temptation to develop battlefield nuclear weapons.

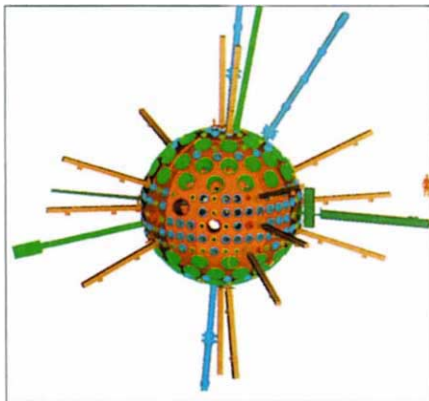
Mitterrand claimed that France's existing nuclear deterrent is sufficient for its defence needs, and could be modernized by the year 2010 using simulation techniques rather than direct testing. But others have expressed concern that a moratorium will make it impossible to achieve the required modernization, such as increased miniaturization and an enhanced capacity of warheads to penetrate defences, as well as to guarantee their reliability.

In 1993, for example, a commission of the conservative-dominated parliament concluded that France's current nuclear weapons would only be an effective deterrent until 2010. But it argued that France lagged behind other countries in simulation techniques, and needed to carry out ten explosive tests to validate its simulation methods, as well as a further ten to test upgraded weapons in the existing arsenal.

France has about 500 nuclear warheads,

including 385 150-kilotonne intercontinental ballistic missiles (ICBMs) carried aboard submarines and 18 others housed in fixed silos on the Plateau d'Albion. It plans to modernize this arsenal, in particular by introducing the M5 ICBM for the next-generation Triumphant-class submarines and the ASLP air-to-surface missile for the Rafale aircraft.

Jacques Chirac, the Gaullist candidate in the presidential election, has taken up the parliamentary commission's conclusions,



Test chamber: 1.8 million joules in a flash.

saying that France may need to carry out more tests, adding that it should also set a date for a definitive stop. His rival, the socialist Lionel Jospin, has called for a permanent test ban.

Whatever the outcome, the decision to build the new laser marks France's biggest step towards using computer modelling and simulation to modernize its current nuclear deterrent while reducing the need for testing in the future. It is part of the wider PALEN programme (Preparation for Limitation of Nuclear Tests), approved in 1993, on which FF900 million was spent last year.

The facility will be built at the Centre d'Etudes Scientifiques et Techniques d'Aquitaine (CESTA) in Le Barp, 30 km from Bordeaux. The research centre is one of the six operated by the Military Applications Division (DAM) of the CEA. The machine will be built between 1996 to 2002

and, according to Daniel Wisshaupt, the manager of the programme, will reach full power by 2005.

The main laser will be 60 metres high and will cover the area of two football fields. A labyrinth of mirrors will reflect light from 240 lasers into a spherical test chamber, 10 metres in diameter. This will contain at its centre a supercold pellet, a few millimetres in diameter, of deuterium and tritium, two isotopes of hydrogen.

During a flash lasting a few nanoseconds, the lasers will unleash 500 terawatts on to the walls of the chamber. This will create a barrage of X-rays that will compress the pellet, heat it to 100 million degrees, and trigger a process of sustained thermonuclear fusion that will increase the temperature to 400 million degrees.

The physical conditions, such as temperature, density and pressure, produced by this miniature thermonuclear explosion will be similar to those in an exploding hydrogen bomb, allowing study of implosion, the acceleration of particles and absorption and emission patterns of nuclear radiation.

Although the energy represents several hundred-thousand times the output of a conventional nuclear reactor, the lights on the Champs-Élysées will not dim each time the laser is fired. As in a camera flash, the energy would be stored up and then released over a very brief period. Indeed, while the power of 1.8 million joules is enormous for a laser, it is comparable to the energy content of a 12-volt car battery.

But if the physics is relatively straightforward, the politics are not. Both the US and French decisions to build such lasers have been interpreted by some as evidence that the two countries want to circumvent a test ban. Steven Dolley, for example, research director at the Nuclear Control Center in Washington, disputes US claims that simulation is needed to improve the safety and reliability of weapons. Dolley argues that such issues have nothing to do with nucleonics, but involve the conventional high-explosive part of the weapons. Lasers, he claims, could be used to design new weapons, and increase the risk of proliferation.

A senior official at DAM agrees that the ban has made it "necessary to rethink everything". But he argues that building the laser will not encourage proliferation, as only states that have already carried out tests will be able to benefit from the simulations.

Although the new laser will also provide physicists with a new toy for studying how stars shine, he says it is unlikely that France will investigate the laser as a means of harnessing thermonuclear power for electricity production. It has committed itself to the magnetic confinement techniques used in tokamaks, and cannot afford to pursue laser experiments in parallel.

Declan Butler

Chinese students abroad face cash deposit

London. The Chinese government has increased efforts to slow down its brain drain by insisting that students intending to study abroad on government scholarships place a cash deposit worth half the scholarship before they depart. The money will be refunded only when they return.

The move was reported last week in the Beijing newspaper *Youth Daily*. Of an estimated 200,000 students to have left China since the start of the 1980s to study abroad, just 70,000 have returned.

In addition to the cash deposit, the government, according to the report, will insist on recording the names and addresses of two 'guarantors' for each student who will be traced and questioned should the student decide not to come back to China.

Previous measures taken by the Beijing government to dissuade students from overstaying abroad include insisting families of married students remain in China and asking European and US interior ministries to refuse work permits. □

Increase for research funds leaves Austria below EU average

Munich. Austria's researchers have been given priority in the 1995 budget — without even having to ask. Compared to an overall increase in the national budget of 2.8 per cent over 1994, research funding will increase by 6 per cent, to 2.89 billion Austrian schillings (US\$295 million).

The Fonds zur Förderung der Wissenschaftlichen Forschung (FWF), which provides money for university research, will receive a 10 per cent increase, to ÖS653 million.

While the average expenditure by European Union (EU) states on research is around 2 per cent of gross domestic product (GDP), Austria's is only 1.6 per cent. All parties want to see this increase. But, despite the increase in cash next year, research spending as a proportion of GDP is expected to fall because of high predicted growth, and the fact that industry is not expected significantly to raise its investment in research.

Rudolf Scholten, the science minister in the coalition government formed last January, said that, even though it is acceptable, the increase is "not generous". Arnold Schmidt, president of the FWF, puts the good fortunes of Austrian research in a more gloomy international light. "Everyone knows that our science budget is too low compared with, say, Germany, which is always an important yardstick for us", he says.

He points out that the Deutsche Forschungsgemeinschaft, the German research agency which is equivalent to the FWF, receives twice as much funding per capita of the general population as the FWF. It also has a guaranteed 5 per cent annual increase over the next few years. "Our ten per cent rise is OK, but we still have a lot of catching up to do", he says.

Scholten has announced plans to streamline research organizations in order to ensure a more efficient use of funds. He argues that Austria has too many small research groups, and that money cannot, therefore, be concentrated on priorities.

He also plans to establish a special fund to provide direct support to young scientists. This is particularly important because the rigid Austrian system of promotion tends to give established professors excessive control over the careers of their young collaborators.

The 1995 budget also provides an above-average 5.1 per cent increase in the general budget for universities, and allocates ÖS800 million to the EU's fourth Framework programme, which is now open to Austrian scientists following Austria's admission to the Union.

Alison Abbott

Swedish council is dismissed amid claims of malpractice

London. The entire governing body of Sweden's state Medical Research Council (MFR) was dismissed by the government last week, amid controversy in the media over the allocation and use of research grants, partly a result of the relatively small size of the country's research community.

The 11-member council is responsible for distributing 330 million krone (US\$46 million) for medical research every year. The dismissal of its governing council follows a series of public accusations of malpractice, culminating last week with allegations that one of the council's long-serving members had awarded himself SEK4.2 million for research, some of which never took place.

Both the Swedish government and the council have ordered separate investigations into the affair, expected to be complete before the appointment of new council members — four of whom are chosen by the government, and the rest elected from Swedish medical researchers — on 23 May.

But the reconstituted council will have to do without the services of one of the country's leading scientists. Håkan Eriksson, a professor of reproductive endocrinology at the Karolinska Institute, resigned last Friday (28 April) after 18 years as assistant secretary of MFR's secretariat, apparently in protest at the way the government had bowed to media pressure in dismissing council members.

Olof Edhag, deputy director general of the National Board of Health and Welfare in Stockholm and one of the council members to be dismissed, agrees, saying that the MFR affair had become a victim of "too little analysis and too much vendetta".

The newspapers, he says, had sensed there was something wrong, but "were unable to properly evaluate the issue". Nonetheless, he adds, the government's decision to "exchange" MFR's council, was the best course of action. It is important that MFR's reputation in the eyes of the public be maintained.

The latest controversy centres on Rolf Öhman, professor of psychiatry at the University of Lund. He has been a member of MFR's Council for 12 years, and for the past three has chaired the subcommittee that allocates funds for research in psychiatry.

Recently, the Swedish daily newspaper, *Svenska Dagbladet*, the nation's second-largest selling daily was handed a 250-page dossier on the psychiatry subcommittee's activities, by another psychiatrist at the university, Hans Bendz.

According to reports in the newspaper, the dossier allegedly shows that Öhman's committee apparently handed out SEK4.2 million in research funds between 1982 and

1994 for projects headed by Öhman himself. Some projects are alleged not to have existed, while others never got off the ground.

Öhman is also said to have submitted research grant applications on behalf of people who were unaware of their names being used. Two such applications were uncovered by *Svenska Dagbladet*. According to a journalist on the paper, on one application, "at least three of the 10 names listed as Öhman's collaborators" had not been told that their names were included.

Öhman has admitted in an interview with *Svenska Dagbladet* he may be partially at fault. But added that he was the victim of a system in need of reform. He has also defended his decision not to initiate research earmarked for funding, on the grounds that he had been too busy with other projects. He told the newspaper that he had, in fact, handed back SEK1.7 million that had not been used.

The allegations against Öhman, however, are the latest in a series of public controversies to hit the research council. Last year, it was learnt that the recipients of a SEK28 million grant for genome research were indirectly associated with members of the subcommittee that handled their application. Despite criticism from Sweden's attorney general, the council refused to apologize.

Later the same year, two women researchers published findings suggesting that the council discriminates against women in appointing university assistant professors. In the national debate that ensued, Sweden's education minister Carl Tham joined calls for MFR to be censured by suggesting that women be appointed to senior positions "even if there are better qualified men" until their numbers equalize.

Both incidents have turned the spotlight on the MFR, and Sweden's medical researchers could have done without the resulting embarrassment. Many admit knowing of problems at MFR long before the current furore; one scientist at the Karolinska Institute, for example, says that, with a relatively small number of researchers — Sweden has only six universities — members of grant-approval committees often find themselves facing their own research applications. "The usual thing was to go out of the room when your own application came up," he says.

But the scientists blame "shallow and exaggerated press reporting", as well as the refusal of the research council to admit past mistakes, as having led to the present state of affairs. Many feel the issue has been badly handled. "It is very unfortunate for the whole of Swedish science," says one.

Hsan Masood

Court blocks move of Arizona telescope

Washington. A US appeals court has backed the ruling of a district court in Tucson, Arizona, that the Forest Service exceeded its authority when it decided a new telescope at the Mount Graham International Observatory should be built 1,300 feet from its originally proposed location.

The 2:1 decision by the US ninth circuit court of appeals has upset officials at the University of Arizona, who point out that the proposed move was intended to assuage criticism that original building plans for the Large Binocular Telescope would disturb the habitat of the indigenous Mount Graham Red Squirrel. The move was made only

at the request of the biologists who had been monitoring the squirrels.

Construction work on the \$60-million telescope has been in abeyance since May 1994, when a petition to halt the work was filed by a coalition of local and national environmental groups concerned that the observatory not only threatened the future of the squirrel subspecies, but also offended local Apache tribes who consider the mountain to be sacred.

The petition charged that the move — which the Forest Service claimed would reduce the impact of the observatory on the squirrels — violated the terms of the Arizona — Idaho Conservation Act, which sets out the conditions under which the observatory could be built.

In July last year, a district court judge supported the petition, ruling against the Forest Service (see *Nature* 370, 407; 1994). The service subsequently appealed against the decision to the circuit court of appeals. But last week, the latter court upheld the original ruling.

The main basis of the ruling given by the two judges who issued the majority opinion, was a sketch of proposed locations for the first three telescopes — out of a total of seven ultimately envisaged — contained in a 'biological opinion' submitted in 1988 by the Fish and Wildlife Service.

The judges agreed with the petitioners that the conservation act intended the telescopes to be placed in the locations in that sketch, and that the Forest Service did not have the authority to circumvent Congress by moving a telescope, even if it would benefit the squirrels.

But in a strongly-worded dissenting opinion, the third judge, Judge Cynthia Holcomb

Hall, disagreed. She claimed that minor adjustments in the locations of the telescopes could be requested by the Forest Service, which is responsible for ensuring that the impact of the observatory on the squirrels is minimized.

Hall pointed out that the majority opinion concedes that three structures have already been moved for this reason — one by 750 feet. There were no objections from environmental groups to the previous moves, and Hall claimed that moving the new telescope would be consistent with these precedents.

Peter Strittmatter, director of Steward Observatory, the leading partner in the development of the Mount Graham International Observatory, admits that the decision has set back the schedule for construction of the telescope.

But he adds that plans are now being drawn up to place the telescope in the location originally proposed in the 1988 biological opinion. Meanwhile, Strittmatter says he expects the University of Arizona to request a reconsideration of the decision.

Environmentalists' groups, which had challenged the decision to move the telescope on the grounds that it broke the agreement in Congress — and have already objected to trees on the mountain being removed by the university in preparation — have welcomed last week's ruling.

But Strittmatter continues to argue that locating the observatory at the original site would have very little impact on the squirrels, and says that the new site would have even less. He points out that the total area devoted to the telescopes is only 8.6 acres out of a total squirrel habitat or more than 10,000 acres.

Leslie Sage

Questionnaire row returns to the courts

Tokyo. A nine-year dispute between faculty members of Hong Kong University over the legal rights to the contents of an epidemiological questionnaire is about to re-enter the colony's courts. On 18 April, the Hong Kong High Court ordered a judicial review of a recent internal inquiry by the university that cleared T. H. Lam, of the university's community medicine department, of charges of plagiarism (see *Nature* 373, 465; 1995).

The review was requested by Linda Koo of the same department and John Ho Hung Chiu, a professor in the department of radiation oncology. In 1992, Koo and Ho won a court case in which Lam had been charged with copying without their permission parts of a questionnaire that they had used for a cancer survey in the early 1980s.

The verdict was upheld by the Court of Appeal the following year. But in January this year the university rejected the court decision. After its own internal inquiry of Koo and Ho's claims against Lam, it cleared Lam of all charges of misconduct.

The High Court will now judge whether the university acted fairly in its internal inquiry. Koo and Ho claim that the inquiry was one-sided, as they were excluded from the hearings, and want the court to quash the university's report and prevent its further publication.

A university spokesman said on Monday that the university is unable to comment on the judicial review of its inquiry because the matter is *sub judice*. Koo says she expects a court hearing within the next few months.

David Swinbanks



Koo: expects a court hearing soon

Chiron expands gene therapy efforts

San Francisco. Chiron Corporation, based in Emeryville, California, and one of the fastest growing US biotechnology companies, geared up last week to become a major player in gene therapy with the \$95-million purchase of Viagene Inc. of San Diego, California.

Calling gene therapy a very promising technology for drug development, Chiron officials said they had been attracted by Viagene's viral and nonviral gene transfer technology, its \$6-million manufacturing facility and its clinical experience.

"We believe that gene therapy is an important enabling technology that will yield innovative health care products across a broad spectrum of indications," said William J. Rutter, chairman of Chiron.

Chiron already has links with Progenitor Inc. of Columbus, Ohio, which specializes in nonviral vectors, and Ribozyme Pharmaceu-

tics Inc. of Boulder, Colorado, which has developed gene expression technology. Chiron and Viagene have collaborated since 1983, when Chiron purchased 17 per cent of the smaller company for about \$20 million.

Viagene has launched eight phase-I human clinical trials, and late last year began phase-II HIV trials with 190 patients. The company has focused on several types of cancer, HIV, haemophilia and hepatitis B. A joint trial with Chiron is currently testing gene therapy using interferon- γ in tandem with interleukin-2 for advanced metastatic melanoma.

According to Marc Schneebaum, senior vice president and chief financial officer of Genetic Therapy Inc., one of Viagene's leading competitors, Chiron's move illustrates the growing interest in gene therapy technology by large biotechnology and pharmaceutical companies.

Sally Lehrman

Physics centre head says he will follow Salam's example

Trieste. The International Centre for Theoretical Physics (ICTP) in Trieste, northern Italy, has completed its long search for a new director. Miguel Virasoro, a theoretical physicist born in Argentina, will next month take up the position vacated at the beginning of last year by the centre's founding director, Nobel prizewinner Abdus Salam.

Virasoro, who is 55, left Argentina in the mid-1970s, and took up a chair in theoretical physics at the University of Rome in 1982. A frequent visitor to the Trieste centre, he was chosen last month by the heads of the International Atomic Energy Agency (IAEA) and the United Nations Education, Scientific and Cultural Organization (UNESCO) from a short-list drawn up after the withdrawal of the sponsoring organizations' original choice, Praveen Chaudhari from IBM's Thomas Watson Research Center in New York (see *Nature* 373, 182; 1995).

Despite high hopes that the new director, like Chaudhari, should be from a developing country, the appointment of Virasoro, who now holds Italian citizenship, has been widely welcomed. "He is a first-class scientist," says ICTP's acting director, Luciano Bertocchi. But Virasoro will find he has a hard act to follow, as the whole shape of ICTP — and, indeed, of science in Trieste — was moulded by Salam, who is still held in high esteem by the centre's staff.

ICTP was set up in 1964 under the auspices of the IAEA to train theoretical physicists from developing countries. From modest beginnings, the centre has grown enormously and now welcomes about 4,000 visiting scientists every year. It operates on an annual budget of more than US\$20 million, most of it provided by the Italian government.

Despite Trieste's relative isolation, the centre has helped to attract other scientific institutes to the city. Situated on the border with Slovenia, Trieste was once the major

port of the Austro-Hungarian empire, but over the past century has lost both economic and political importance.

Despite a long tradition in the humanities — the city had no scientific tradition until the arrival of ICTP — it now hosts Italy's only postgraduate school, the Scuola Internazionale Superiore di Studi Avanzati (SISSA), as well as the International Centre for Genetic Engineering and Biotechnology, and Elettra, a state-of-the-art synchrotron facility that began operation this year.

Salam also helped to set up three independent research centres — in materials, chemistry and environmental science — under the auspices of the United Nations Industrial Development Organization (UNIDO). The core staff of these centres, which were launched in 1988, currently occupy the seventh floor of an ICTP building, known as "seventh heaven".

But without Salam's personal involvement, the original plan for the expansion of these centres, whose activities were intended to complement the more theoretical work of the ICTP, is unlikely to be put into effect. UNIDO wants the centres to become more industrially-oriented, as does the Italian government, which currently pays for them, but which would like industry to share the cost.

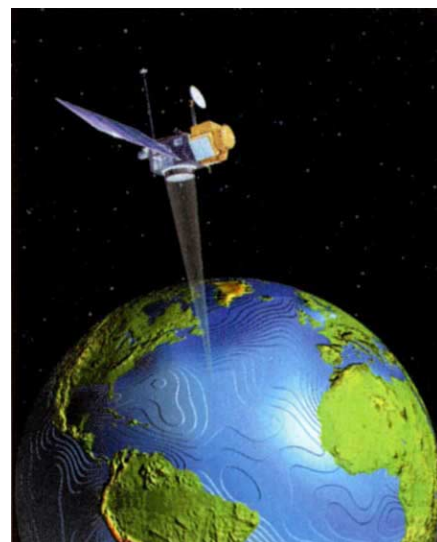
In recent years, ICTP's growth has created problems for the IAEA, which is concerned that the content of the centre's courses has extended well beyond the competence of the agency required to approve them. A few months ago, the Italian government agreed that responsibility for the administration of the centre be transferred to UNESCO — previously a junior partner — from next year.

The transfer has been welcomed by ICTP staff, in particular because it will result in better employment conditions. Under the IAEA, most staff contracts lasted just one year; now all staff will be on five-year renewable contracts. The new arrangement also transfers more administrative autonomy to the centre, increasing its direct control over its budget.

But it will also add to uncertainty over ICTP's future direction, as it is not yet known what influence UNESCO will want to have on the centre's range of activities. Virasoro says that he is keen to continue the tradition established by Salam, and told ICTP staff last week that he is not planning any changes in the centre's direction.

At least the long-term financial future looks secure. An Italian law passed in January guarantees the ICTP at least 20 billion lire (US\$11.8 million) a year until further notice. Before that, funding had only been secure up to 1998.

Alison Abbott



Topex: measuring the height of oceans.

NASA 'to pick France' for ocean satellite

Washington. The US National Aeronautics and Space Administration (NASA) is expected to choose a French-built radar altimeter rather than a US-manufactured instrument to fly as part of the agency's Earth Observing System (EOS) in 1999.

The altimeter, modelled on one launched on the US-French Topex-Poseidon spacecraft in 1992, is likely to be selected in preference to the US Navy's proposed Geosat Follow-On 2 (GFO-2) proposal. The two instruments provide similar information on sea-surface topography, and were similarly priced. But NASA officials have been keen to involve the French as partners on the EOS project.

William Townsend, deputy associate administrator for NASA's Mission to Planet Earth programme, says "the most important thing to us at this juncture [is] the international collaborative aspect". The Topex-Poseidon follow-on option is expected to cost more than \$300 million, half the money coming from the French government.

A study by the National Academy of Sciences concluded that either of the GFO-2 or Topex/Poseidon instruments could meet the EOS project's needs if flown in the Topex orbit. In terms of science return, "one would be as good as the other", says Townsend.

In addition to providing EOS scientists with sea-surface data, the Topex-Poseidon altimeter will serve the US Navy as a backup to GFO-1, due to be launched next year.

In the event of an international conflict, the Navy has been assured that data from the instrument could be encrypted for security reasons. Townsend says they would still be made available to scientists, and that even though it would be on a slightly delayed basis, that would not affect the usefulness of the data for global change research.

Tony Reichhardt

Corrections

Claude Allègre was described in a news story on the French presidential elections as head of the Institut du Physique du Globe at the University of Paris VI (*Nature* 374, 666; 1995). Having occupied this post from 1976 to 1986, he is now head of the department of geochemistry at the institute, and president of the Bureau de Recherches Géologiques et Minières. The current head of the institute, which is not part of the university, is Jean-Louis Le Mouél.

A recent news story on South Africa (*Nature* 374, 487; 1995) should have said that the science budget will be increased by 4.8 per cent next year, not 1.8 per cent.

Death of British space science?

SIR — Ken Pounds, the chief executive of the UK Particle Physics and Astronomy Research Council (PPARC) responds (*Nature* 374, 491; 1995) to letters from the space astronomy community angry over a British withdrawal from the Integral space mission by shifting the blame for the present débâcle onto his predecessors at the Science and Engineering Research Council (SERC). A more important issue is what is done to resolve what is surely a matter of immense concern for a nation with a great heritage in space science.

Pounds says that "PPARC is actively working with its space partners to find additional funds for exploitation of future spaceflight opportunities". The PPARC policy (see *Nature* 372, 712; 1995) relies on the introduction of massive increases in efficiency to allow a 25 per cent reduction in member state contributions. The figure that would then be returned annually to the United Kingdom is that required to rebuild a domestic space budget which, as Pounds indicated, somehow disappeared in the final days of the SERC. I would like to see this policy buttressed by PPARC declaring that it will ring-fence any returned money for space. There must be a guarantee that if the massive changes targeted by the British are agreed, the domestic problem will not recur. PPARC has little to fear from such a step as I understand that it does have a guarantee from its masters at the Cabinet Office that money returned to PPARC from a reduced subscription to the European Space Agency (ESA) subscription will remain with PPARC.

Ironically, some of the efficiency gains promulgated by the British are already being implemented to get the Integral mission off the stocks without the United Kingdom. Large savings in payload cost (perhaps more than 15 per cent) are needed to keep Integral on track. The agency (and PPARC's partners in ESA) are being imaginative to achieve the necessary savings and they are certainly picking up on ideas put up by the United Kingdom.

Pounds agrees with his British critics that "it is vital that PPARC finds some way to ensure that the Integral débâcle does not recur with subsequent missions". I could not agree more. The British scientists looking to collaborate in Rosetta, the cornerstone cometary mission to be launched early in the next century, are already being treated with scepticism by their partners.

Could not PPARC improve its credibility by not only making its domestic policy consistent with what it has enunciated in ESA but also by being seen to make efforts domestically to bring its own house into order as a first step? Without UK

credibility, its partners might be tempted by the option of proceeding to enjoy the benefits of efficiency brought by UK pressure but without coming near bailing out the United Kingdom.

None of this should get in the way of the fact that this year is a year of triumph for the ESA space science programme with the launch of six spacecraft, all of which have massive British involvement. The investments made, consequent on the fact that British space scientists have won most of their funding battles of the past decade, are just coming to fruition. If PPARC cannot find funds for Integral and Rosetta, the death knell for British space science may have sounded, but it is going to be a long and glorious death.

David Southwood

(Chairman, ESA Science Programme Committee)

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Diverging trends, intersecting issues

SIR — The argument, presented in a leading article (*Nature* 374, 199–200; 1995) that the world should wait two, five or even more years before formulating a strict protocol on anthropogenic greenhouse gas emissions is an understandable one. But this does not mean that individual nations, or even groupings of nations such as the European Union, should not set themselves their own goals straight away.

While it may well be prudent to call for a pause so as "to get the whole world on side", it is perhaps erroneous to suggest — as you do in conclusion — that the reason is analogous to "unilateral disarmament being a fruitless policy". Unilateral interim action on anthropogenic climate change by those nations that can (primarily the developed nations) will do three things.

First, the developed (OECD) countries consume by far the most fossil fuels, both nationally and per capita, so that any progress in reducing emissions here has a good chance of lowering overall emissions in the short term. Second, moving to an economy less dependent on finite fossil fuels should provide those nations with greater security and potentially greater economic stability.

Third, in moving to an economy that is energy efficient and/or not based on fossil fuel, more appropriate technology will have been developed to a level where it might perhaps be used in countries yet to have made the transition. (There is a

fourth point in that an example will have been set: not one for environmental ethics — though that will be the case — but an example in practicality.)

So 'yes' to your call for caution at Berlin, but also 'yes' to unilateral action.

Jonathan Cowle

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Science and democracy

SIR — You rightly highlight the need for public understanding of science (*Nature* 374, 291–292; 1995), but miss one of the most important reasons why it is desirable — democracy. It cannot be justified that, in nations that regard themselves as democratic, the population at large has little or no effective political control over the directions that research takes, and the uses to which scientific discoveries are put. Where we are concerned with the regulation of matters as diverse as embryo research, the use of transgenic plants in the field, or the safety (or otherwise) of nuclear power, it is surely in all our interests that as many as possible have knowledge that enables them to be involved in the decision-making process.

On the other hand, a properly informed and scientifically literate population would be able to enter into a rational discussion with the scientific community; this is surely the way in which the regulation of sensitive or controversial areas of research should be determined. The alternative is self-regulation by an unaccountable élite (a formula that has been singularly unsuccessful in other walks of life, for example the City of London), breeding resentment and alienation as a consequence.

The research community must not be a law unto itself; if it is seen to avoid giving the public the trust it deserves, it will in turn lose the trust of the public, and will deserve to do so.

Robin Walters

(Secretary, Scientists for Labour)
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Cold War against the Vatican

An error was introduced in the editing of the letter from Serra and Neri (*Nature* 374, 589; 1995).

The last sentence of the first paragraph should have read: "Nothing could be further from the correctly interpreted facts of human reproduction and development than Godfrey's statement that "... we should envisage human persons coming into being ... as each bright day is imperceptibly created during dawn." □

The case for a great many journals

General opinion reacts badly against new journals, but on the grounds that there are too many already. But any issue of any journal contains something of more than passing interest.

WHY are there so many journals? The simple answer is that there are so many people working at research, all of whom must publish regularly, so that there is an endless demand for pages that will carry text. And because there are limits to the growth of individual journals, perhaps determined by economic considerations or even by editors' proper sympathy for the amount of material their readers can handle in one package, it follows that there must be a secular increase in the number of separate journals.

That is one side of the coin. The other is that, however far down in the pecking-order of selectivity a journal may be, it is very rare that its consecutive issues fail to include something of wide general interest. Inveterate browsers in the libraries know that already. What follows is an account of one browser's findings in a handful of journal issues reaching this office on a recent day, and selected (without knowledge of the purpose) by somebody else.

First, there is *Physics Letters A*, which happens to include a discussion of an argument originally put up by Per Bak and K. Sneppen (*Phys. Rev. Lett.* **71**, 4087–4090; 1993) to the effect that the episodic character of the fossil record, called punctuated equilibrium by S. J. Gould, may be explicable by a simple mathematical model. The model (see *Nature* **371**, 197; 1994) supposes a one-dimensional lattice and the assignment to each vertex of a random real number (between 0 and 1).

Each point represents a species and its random number its fitness. The "ecosystem" then evolves by the simple rule that the smallest number in the chain and its two nearest neighbours are replaced by another random number in the chain. This rule creates a bias against small numbers; if the lattice is infinite, they will all grow to be bigger than some critical limit. But in that condition, the lattice is unstable. If, at any step, the smallest number and its two neighbours should by chance be replaced by numbers less than the critical value, there will be an avalanche of small numbers, or an extinction.

Peter Grassberger from the University of Wuppertal (*Phys. Lett. A* **200**, 277–282; 1995) now says that it seems "very unlikely" to him that such a static model could represent a real ecosystem; in another context, he uses the slighting phrase "a physicist's toy model". He nevertheless suspects that it may be a model that

"sooner or later" will find an application. He even considers the question of whether the model is a special case of the model system known as "directed percolation", originally devised to account for collisions between hadrons, but now more properly applicable to epidemics and the spreading of damage. He concludes that it is not. So the question remains as to what the model really applies.

Then there is the matter of the claimed new allotrope of carbon ("white carbon"?), prepared by Sei-ichi Tanuma and Andrei Palnichenko at the Iwaki Meisei University in Japan (*J. Mater. Res.* **10**, 1120–1125; 1995). Already, of course, there are diamond and graphite, not to mention C_{60} . Crystals of the new material are made by condensing carbon vapour to room temperature, and form either rhombohedral or hexagonal crystals (up to a millimetre across) with their principal axes perpendicular to the substrate. They are fibrous, and white in colour, with a density of 1.46 g cm^{-3} , whence the etymologically careless name of "carbolite".

The explanation? Think of a string of carbon atoms held together by double bonds (called cumulene), perhaps made by the dehydrogenation of acetylene. This is one-dimensional carbon, or carbyne. Then stack these molecules parallel to each other and you have a material that forms a crystal held together by lateral van der Waals forces between the chains. The chains themselves are staggered, with four atoms in a line followed by four offset by a small degree. The interchain separation is 0.344 nm in the hexagonal crystal and 0.337 nm in the rhombohedral variety. It is a good tale that will be more persuasive when there is an X-ray structure.

Now AIDS in Thailand. This week's *Lancet* carries a study of the degree to which couples in which the male is infected with HIV carry similar serological types of the virus. Thailand stands out in the annals of the AIDS epidemic by the speed with which the disease has spread in the past few years. Dr Chaiyos Kunanont (from the Thai Department of Communicable Disease Control) and colleagues now say that those who have acquired the infection by heterosexual intercourse are predominantly infected with the A serotype of HIV-1 (to the extent of 92 per cent), while those whose infection was acquired by intravenous injection were more likely (74 per cent) to be serotype B (*The Lancet* **345**, 1078–1082;

1995). This finding supports the view that the rapid heterosexual spread of AIDS in Thailand is a function of a readily transmissible strain of HIV-1, presumably only recently introduced. That implies that the country's AIDS status is now in transition; serotype B will in due course be swamped by serotype A. And elsewhere? Should not sub-typing of HIV be routine?

The journal *Applied Physics Letters* is a very different kettle of fish, in reality the place in which to look for novel designs for semiconductor lasers, the devices that make our laser-printers work. But a group from Stanford University is worried by a more practical problem: how to know when the growing thickness of a layer of material being deposited on a silicon base has reached the thickness intended? J. Pei et al. have figured out that this can be done, in real time, simply by measuring the velocity of sound with vibration perpendicular to the growing silicon chip (*Appl. Phys. Lett.* **66**, 2177–2179; 1995).

How can that be done? Simple. Support the silicon chip on two identical quartz rods pointed at the end, one of which is a source of ultrasound and the other is capable of receiving it. The transmitting rod is excited at 200 kHz (quartz is piezoelectric) and generates an echo from the chip above, whose receipt can be recorded as a function of time. The difference between that time and that recorded by the second quartz rod gives the travel time along the surface of the chip. A simple experiment with a film being built up by sputtering shows that film thickness can be controlled to a small fraction of a micrometre.

Finally, in the *New England Journal of Medicine* is an encouraging account of a fetal brain tissue transplant into a patient suffering badly from Parkinson's disease (J. H. Kordower et al. **332**, 1118–1124; 1995). The patient died of unrelated causes 18 months after transplantation, allowing those concerned to make a histochemical examination of his brain, and especially the substantia nigra, where dopamine appears to function. The conclusion is that the implanted neurons had survived "robustly". Although the patient had improved after the operation, he nevertheless required medication: he had not been cured but these are early days.

So there is a rag-bag of incidental information, but also a browser's charter. Nobody should complain that there are all those journals.

John Maddox

Interfering with the runners

Scott Brady

In 1939, the New York Yankees baseball player Lou Gehrig ended his career after starting 2,130 consecutive games over 15 years. The sequence, which remains a record, finished only when an athlete legendary for his endurance was inexorably brought down by the most common of motor-neuron diseases, amyotrophic lateral sclerosis (ALS), now more familiarly known as Lou Gehrig's disease.

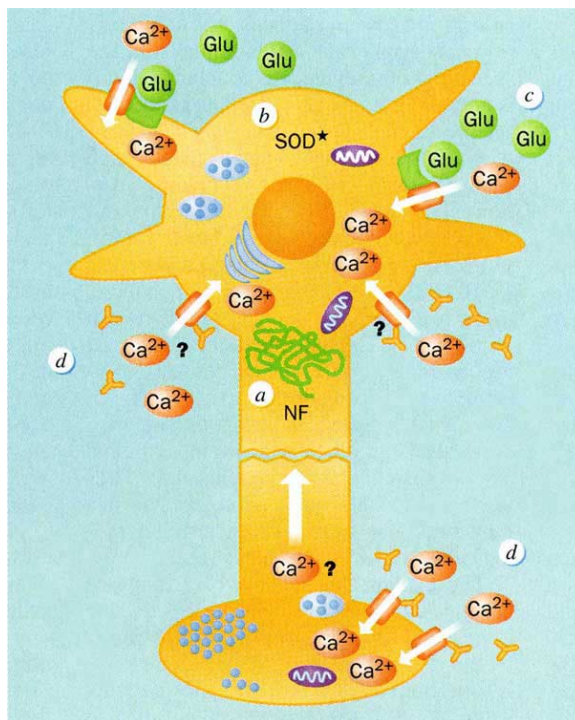
Gehrig's record may finally be beaten this year. The same cannot be said of the disease that bears his name, but since 1992 our knowledge of it has advanced on several fronts. The latest development is to be found in the paper by Collard and colleagues (page 61 of this issue¹). Using a transgenic mouse model of ALS, they make a strong case that the aberrant accumulation of neurofilaments in motor neurons, and consequent disruption of axonal transport, can be responsible for neuron death.

More than 70 neurological disorders, affecting 1 in 20,000 people, involve specific loss of motor neurons². Some result from defective enzymes, environmental neurotoxins or infective agents, but the causes of most are unknown. ALS itself is typically of adult onset, and involves a progressive degeneration of descending motor pathways that is generally fatal within five years of diagnosis; 90 per cent of cases are sporadic and of uncertain aetiology.

The most notable events of the past three years have been the provisional identification of four distinct mechanisms that lead to specific loss of motor neurons. First, in 1992, Rothstein *et al.*³ reported that ALS patients have reduced capacity for glutamate transport in the central nervous system, implicating excitotoxicity in cell death. Prolonged activation of glutamate receptors produces an influx of Ca^{2+} and neuronal degeneration⁴. Consistent with excitotoxicity, chronic inhibition of glutamate uptake leads to motor-neuron death *in vitro*⁵ and there is evidence of loss of glial glutamate transporters in motor areas of ALS patients⁶. The mechanism of selective loss is unknown, but neurons in transporter-deficient areas would be vulnerable to excitotoxic pathology.

Second, in the following year the Cu/Zn

superoxide dismutase gene (*SOD-1*) was shown to be altered in some cases of familial ALS (ref. 7). Several autosomal dominant alleles leading to disease have been identified, but only a quarter of familial ALS cases are traceable to mutant *SOD-1* genes. Although this suggested that oxidative damage might be responsible for motor-neuron death⁴, transgenic



Mechanisms that can lead to motor-neuron death in ALS. *a*, Accumulation of neurofilaments (NF) in the proximal axon prevents delivery of mitochondria and other essential components to the axon. *b*, Mutant forms of superoxide dismutase (*SOD**) lead to an increase in cell-body vacuolation, mitochondrial degeneration and, possibly, to neurofilament accumulation. *c*, Loss of glial glutamate transporters results in elevated levels of extracellular glutamate (Glu), activating motor-neuron glutamate receptors to produce Ca^{2+} influx and excitotoxicity. *d*, Antibodies against Ca^{2+} channels (Y) lead to increased Ca^{2+} influx, accumulation of vesicles in the presynaptic terminal and cell death; Ca^{2+} entry by this route may occur at the level of the cell body or through the axon. Each of these pathways could itself kill motor neurons, but synergistic interactions might also be involved.

mice expressing mutant *SOD-1* develop ALS-like pathology despite having near-normal enzyme activity^{8,9}, and more than 95 per cent of ALS patients have wild-type *SOD-1*. In all, *SOD-1*-linked familial ALS seems to stem from some unknown cytotoxic gain-of-function^{8,9}.

Concurrently, autoantibodies to Ca^{2+} channels were detected in ALS patients¹⁰. These antibodies recognize several types of Ca^{2+} channel, inhibiting currents through L-channels but increasing them through P-channels¹¹. Such currents could

lead to neuronal degeneration through mechanisms similar to excitotoxicity, and the antibodies were cytotoxic *in vitro*¹². These results pointed to a third, autoimmune, component of ALS.

Finally, indications of a fourth mechanism came from development of ALS-like pathology in transgenic mice overexpressing normal and mutant neurofilament subunits¹³⁻¹⁵. Not all transgenic mice develop ALS-like pathology¹⁶, but motor neurons are preferentially affected in those that do. Neurofilamentous accumulations in cell bodies and proximal axons are a hallmark of ALS, so these transgenics implied that disruption of neurofilament transport might cause motor-neuron death.

A variety of pathologies with different underlying causes are also associated with disruptions in axonal neurofilaments. Among them are diabetic neuropathy, Charcot-Marie-Tooth disease, and the effects of selected neurotoxins. Some have an associated increase in neuronal degeneration, but others lead to impaired function without a substantial loss of neurons. In most cases, however, pathological changes in neurofilaments are most apparent in large motor neurons, consistent with the fact that neurofilament expression is highest in these cells¹³⁻¹⁵.

Given that such diverse aetiologies have such similar consequences, the first lesson may be that neurodegenerative diseases are clinically defined by loss of specific neuronal populations, not by underlying mechanisms. Neurons are large, complex cells with unique interdependence on their targets and partners. Protein synthesis is restricted to cell bodies, and the metabolic needs of maintaining axons that, in humans, may be more than a metre long, place special demands on neurons. Both anterograde and retrograde degeneration can result from disruption of trophic relationships. Motor neurons affected by ALS are among the

largest in the body, and extension of their axons into the periphery makes them especially vulnerable.

The observations of Collard *et al.*¹ provide a second lesson and illuminate the various mechanisms that lead to motor-neuron disease. In a transgenic mouse model of ALS produced by overexpressing the gene for human neurofilament heavy-subunit, the amount of material transported into axons is reduced, consistent with the distal atrophy seen in ALS axons. In particular, the number of

Timing is everything

Alan P. Boss

mitochondria detectable in axons is reduced, meaning that axons may degenerate because they lack the metabolic energy to maintain even minimal function.

The implication is that accumulations of neurofilaments seen in ALS impede normal movements of other cellular structures, and that degeneration begins when supplies of new organelles and structures fall below a certain threshold. Other animal models of ALS also exhibit disrupted transport processes. In both glutamate excitotoxicity models⁵ and the *SOD-1* transgenic mice^{8,17}, the cell bodies of motor neurons display vacuoles and degenerating mitochondria. Similarly, autoantibodies to Ca^{2+} channels produce vesicle accumulations in the distal axons of motor neurons¹⁸, possibly reflecting disruption of trophic-factor delivery by retrograde transport. Finally, the Golgi apparatus is often fragmented in ALS motor neurons¹⁹.

Making progress on ALS will depend on getting to grips with its underlying complexity. Not every pathogenic mechanism producing motor-neuron death may disrupt axonal transport, and such disruptions may be secondary to the primary lesion in others. Nonetheless the observations of Collard *et al.* show that disrupting neuronal dynamics can lead to degeneration of specific neuronal populations. The point of disruption may vary with aetiology (mitochondria or Golgi, fast or slow transport, anterograde or retrograde movements, and so on), but it is the size and location of motor neurons that puts them at risk. □

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How long did the earliest phases of planet formation last in the inner Solar System? New measurements of aluminium isotope abundances in primitive meteorites imply that rocky solids were formed in a dusty disk over a period of about 10 million years (Myr). That period of time corresponds nicely to the ages of the oldest newly formed solar-type stars that still show evidence for having planet-forming disks. And theorists are famously adept at inventing new schemes for explaining almost any laboratory result, including a 10-Myr span for the aggregation of rocky minerals. So is the question settled? Research presented at a planetary science conference in March* suggested that this was the case, and that a coherent picture is emerging. But the concerns of a few doubters may yet cause rain to fall on the gathering parade.

Deciding on the chronology of the formation of bits and pieces of the oldest rocks in the Solar System is a necessary step in trying to understand how our planetary system formed. Refractory, centimetre-sized inclusions in the Allende meteorite are the oldest objects yet found, with ages of about 4,566 Myr. Carbonaceous chondrite meteorites like Allende often contain refractory inclusions, but are characterized predominantly by the presence of chondrules, millimetre-sized silicate spherules whose igneous textures are clear evidence of a molten origin. Some chondrules contain relict fragments of refractory inclusions, implying that the chondrules formed after the refractory inclusions. The volatile elements found in chondrules also suggest formation after refractory inclusions, at a time when the protoplanetary disk had cooled significantly. How much later is the big question — refractory inclusions define the starting point of planetary formation, whereas chondrules are thought to represent the bulk of the matter that formed the rocky cores of the planets.

Refractory inclusions which are rich in calcium and aluminium (termed CAIs) often contain more of a key magnesium isotope (^{26}Mg) than is typical of primitive

materials for a given total amount of magnesium. Moreover, the amount of excess ^{26}Mg correlates with the abundance in different mineral phases of ^{27}Al , the stable, dominant isotope of aluminium. ^{26}Mg can be produced by the radioactive decay of ^{26}Al , which has a half-life of 0.7 Myr. These three facts imply that the excess ^{26}Mg was produced by the *in situ* decay of ^{26}Al , which behaves chemically in the same way as ^{27}Al . Because of its short half-life, ^{26}Al makes an excellent chronometer for the earliest phases of



Cross-section of the Axtell carbonaceous chondrite meteorite, showing an abundance of centimetre-sized refractory inclusions surrounded by a host of millimetre-sized chondrules. In spite of being found intermingled in meteorites, these two components are thought to have formed independently in the Sun's planet-forming disk millions of years apart in time. Photo courtesy of S. B. Simon and L. Grossman (Univ. Chicago, Field Museum).

planet formation.

The presence of live ^{26}Al in CAIs requires that no more than about 1 Myr could have elapsed between the nucleosynthesis of the ^{26}Al and the formation of CAIs in the solar nebula. The bulk of the ^{26}Al may have been synthesized in a red giant (AGB) star (G. J. Wasserburg, California Institute of Technology); pre-solar graphite grains contain a potpourri of isotopes (including excess ^{26}Mg) possibly derived from mixing the nucleosynthetic products from a supernova explosion in a massive star (E. Zinner, Washington Univ.).

With the radioactive decay clock ticking, the fresh ^{26}Al must have been expelled from an AGB star during its planetary nebula phase, or by the supernova phase of a more massive star. Surfing along on an interstellar shock wave, the ^{26}Al was injected into a dense molecular cloud that

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*Twenty-Sixth Lunar and Planetary Science Conference, NASA Lunar and Planetary Institute, Johnson Space Center, Houston, Texas, USA, 13–17 March 1995.

got in the way of the shock wave. The shock wave not only injected the live ^{26}Al , but also forced the cloud core into self-gravitational collapse (P. N. Foster, Carnegie Institution of Washington), leading directly to the formation of the proto-Sun and the solar nebula. Alternatively, the ^{26}Al may have been produced in the presolar molecular cloud core by cosmic-ray irradiation (D. D. Clayton, Clemson Univ.), but observations of γ -rays produced by cosmic ray bombardment in the Orion star-forming cloud indicate a cosmic-ray flux that is too small to produce the required amount of ^{26}Al relative to ^{27}Al (R. Ramaty, B. Kozlovsky and R. E. Lingenfelter, *Astrophys. J.* **438**, L21–L24; 1995).

The collapse of the presolar cloud required about 0.1 Myr. Assuming that the proto-Sun was able to form similarly rapidly, CAIs could then be the first solids surviving from the early hot nebula phase (A. G. W. Cameron, Harvard-Smithsonian Center for Astrophysics). The CAIs formed over a period of less than 0.1 Myr, retaining most of their ^{26}Al . However, searches for excess ^{26}Mg in chondrules have generally yielded only upper bounds, not detections (I. D. Hutcheon, Lawrence Livermore National Laboratory), implying chondrules formed so much later (at least 3 Myr) that nearly all of the ^{26}Al had already decayed. Because chondrules are thought to have formed within the dust-rich environment of the solar nebula, this requires that the nebula lifetime was about 10 Myr.

Now the problems start. The CAIs must have been preserved for ~10 Myr and then accumulated along with newly made chondrules to form the carbonaceous chondrites (see photograph). But the CAIs cannot be stored in the solar nebula for so long, because gas drag forces centimetre-sized pebbles to spiral into the Sun within about 0.1 Myr. This disaster leads to the idea of storing CAIs in much larger (kilometre-sized) bodies, with relatively stable orbits. The large CAI-filled piñatas would then have to be broken apart 10 Myr later, releasing the CAIs for incorporation into chondritic bodies (W. R. Skinner, Oberlin College). Another solution is to assert that the chondrules found in carbonaceous chondrites like Allende and Axtell were formed soon after the CAIs and therefore still contained live ^{26}Al , whereas the chondrules in ordinary chondrites (^{26}Al -free) formed some 3 to 10 Myr later (Cameron). The latter hypothesis has the great advantage of being immediately testable. The first results are not overly encouraging: chondrules from the Manych ordinary chondrite show no excess ^{26}Mg , but neither do chondrules from the Kainsaz carbonaceous chondrite (Hutcheon).

Finding a mechanism for melting the chondrules is a headache (see R. Ash,

Nature **372**, 219–220; 1994) that may only become worse after waiting for 10 Myr (J. A. Wood, Harvard-Smithsonian Center for Astrophysics); nearly all of the gravitational energy liberated by the formation of the Sun was lost within the first million years. Also, relatively few solar-type stars still have disks at 10 Myr, but the Sun might just be an oddball.

Finally, the absence of excess ^{26}Mg in chondrules might simply be due to heterogeneity in the initial distribution of ^{26}Al ; after all, some refractory inclusions show little or no excess ^{26}Mg , and this is attributed to heterogeneous initial ^{26}Al (Wood). Spatial heterogeneity has also been inferred for the short-lived isotope ^{53}Mn , based on differing amounts of the

daughter product ^{53}Cr found in chondritic meteorites and the Earth (G. W. Lugmair, Univ. California, San Diego). Initial heterogeneity may, however, be hard to preserve in a turbulent, differentially rotating, protoplanetary disk.

Until these lingering doubts are resolved, we cannot regard the issue of a 10-Myr period of chondrule formation as being settled. There will be no shortage of problems with the CAI-chondrules chronology to address at next year's conference. □

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EVOLUTIONARY BIOLOGY

i'iwis fit the bill

William J. Sutherland

THE i'iwi (*Vestiaria coccinea*) is a Hawaiian honeyeater which, as records from the last century show, used to feed largely by extracting nectar from lobelioid flowers. The i'iwi's long, downcurved bill is well adapted for extracting the nectar from the base of the deep corollas of these flowers, which they pollinate in the process. But lobelioids are no longer abundant, and in a paper in *Conservation Biology*¹ Smith and colleagues describe evidence that the i'iwi's bill is evolving in response to the bird's enforced change in feeding habits.

Lobelioids used to be a prominent component of Hawaiian forests. In the past 100 years, however, a quarter of the species have become extinct and the remainder are rare as a result of habitat changes and grazing by feral ungulates. I'iwi now feed largely upon the flowers of

the ohia tree, *Metrosideros polymorpha*. According to nineteenth-century naturalists, i'iwis were excluded from this tree by the aggressive behaviour of another honeyeater, the 'o'o (*Moho nobilis*). But the 'o'o was extinct by 1900.

Ohia flowers lack corollas and the other honeyeater species that feed upon them have short bills. Smith *et al.* thus predicted that the i'iwi should evolve a shorter bill and they compared measurements of museum specimens collected before 1902 with measurements of live specimens. This analysis showed that i'iwis have undergone statistically significant declines in upper mandible length by about 2–3 per cent, whereas characters such as wing or tarsus length are the same. No such change in mandible length was recorded in a related honeyeater species, the apapane *Himatione sanguinea*, that had not altered its diet.

The classic example of a microevolutionary change in bill morphology is that in one of Darwin's finches, *Geospiza fortis*, on Daphne Major Island in the Galápagos. There the population shows rapid declines in bill depth and width after severe El Niño events, as a result of a short-term fall in the abundance of large seeds and an increase in small ones^{2,3}. The especial interest in the case of the i'iwi, however, is that the adaptations in bill length are a response to extinctions and are likely to be long-lived. □

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IMAGE
UNAVAILABLE
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REASONS

Change of diet — i'iwi in an ohia tree.

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Continental roots go with the flow

Walter D. Mooney

ON page 50 of this issue, Vinnik *et al.*¹ add fresh fuel to a heated geological debate. The question is whether the continents have deep, stable roots that have remained practically unchanged since the Precambrian era, or whether plate motion in geologically recent times has deformed those roots. Vinnik *et al.*, working with seismic anisotropy measurements in the Kaapvaal craton of South Africa, have evidence that the continental root has been sheared by the flow of the underlying mantle. Dating back to the Archaean, 2.5 billion years ago, the Kaapvaal is one of the oldest of the Earth's cratons (regions of crust that have remained almost undisturbed for millions of years).

Seismologists continue to discover uses for late-arriving seismic phases in defining the properties of the Earth's interior. In just the past decade or so, work has progressed beyond refining models of the seismic velocity distribution of the Earth using global seismic tomography. In the process, an important discovery was made: the subcontinental mantle shows seismic anisotropy parallel to the Earth's surface²⁻⁴. This is evidenced by shear-wave splitting, where an incident wave is polarized into two orthogonal directions travelling at different velocities (the phenomenon is analogous to birefringence in minerals).

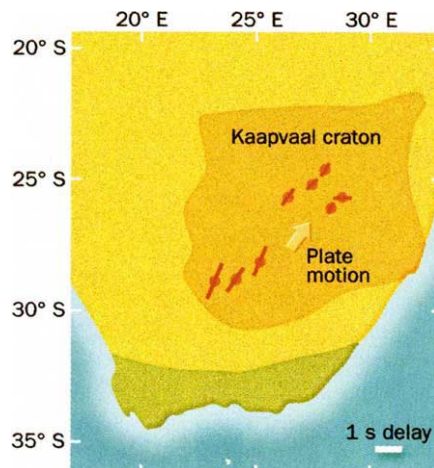
Seismic anisotropy of the mantle is generally taken to arise from strain-induced preferred orientation of highly anisotropic crystals such as olivine. In the oceanic lithosphere, for instance, seafloor spreading aligns mantle olivine crystals with their seismically 'fast' direction perpendicular to the spreading ridge⁵. The challenge has been to work out how, when and where, within the supposedly stable continents, this alignment could have arisen.

Shear-wave splitting, and hence anisotropy within the continental mantle, can be quantified by examining the seismic phase SKS, which travels through the liquid outer core of the Earth as a compressional (P) wave and emerges at the base of the mantle as a shear (S) wave. This phase travels nearly vertically through the mantle, and any shear-wave splitting depends on the magnitude and depth of anisotropy in the subcontinental mantle.

There's the rub. As the SKS phase vertically integrates seismic anisotropy from the base of the mantle to the detector on the surface (a distance of 2,885 km), the depth extent of the anisotropy is ambiguous. It could arise from a near-surface mineral alignment or from deep in the

mantle. There has been a gradual consensus that the anisotropy is concentrated in the upper mantle, as the measured azimuth of anisotropy sometimes changes by as much as 90° at seismic stations separated by only hundreds of kilometres. As SKS phases recorded 100 km apart would have practically the same path in the lower mantle, the anisotropy must be within the upper mantle, that is, above the main mantle discontinuity at a depth of 660 km.

To narrow the range down further, Vinnik *et al.* add the observation that the



Anisotropy in the Kaapvaal craton¹: lines show polarization direction of the fast wave. Their lengths indicate the time lag of the slow wave.

mobility of olivine is temperature-dependent and will be significant only at temperatures over 1,000 °C, corresponding to depths greater than 150 km beneath stable cratons. Combining this with mineralogical arguments⁶ for decreasing intrinsic anisotropy below 400 km, Vinnik *et al.* conclude that mantle anisotropy is localized between 150 and 400 km deep (that is, within the supposedly stable lithospheric 'root' of the Precambrian continent). This depth range is more limited than earlier estimates that only localized mantle anisotropy between 40 and 670 km, with little consensus about where exactly it occurred.

So far so good, but Vinnik *et al.* were looking at the dual question of the depth extent and origin of the anisotropy. Even if most of the anisotropy arises in the upper mantle, it is uncertain whether it dates back to the Precambrian assembly of the continents more than 600 million years ago or is a recent feature caused by deformation of the root as the continental plates drift.

Vinnik *et al.* find that in measurements

taken at eight seismic stations deployed across the Kaapvaal craton of South Africa, the azimuth of the seismic anisotropy is closely aligned with the direction of absolute plate motion for southern Africa since the end of the Jurassic period. So they reject the earlier notion⁴ that seismic anisotropy is primarily a fossil feature of the lithosphere, and argue that it is the result of resistive drag by the mantle. Something similar has been seen elsewhere: the alignment reported for the North American craton³ (by a group again led by Vinnik) is also in the direction of current continental drift.

The Precambrian continental lithosphere was assembled from microplates that collided and coalesced into stable cratonic blocks, much as the large continental plates of India and Eurasia have recently collided. The earlier hypothesis that mantle seismic anisotropy is a fossil feature dating back to the assembly of Precambrian lithosphere⁴ was based on sparser data than the new South African data reported here by Vinnik and co-workers. In the assessment of Vinnik *et al.*, fossil anisotropy in the South African lithosphere should have been indicated by anisotropy that was everywhere parallel to the azimuth of microplate assembly of the Kaapvaal craton. Their observation that, instead, the azimuth of anisotropy is uniformly aligned with the direction of absolute plate motion makes a mobilistic interpretation more likely to be correct.

Nonetheless, there is little doubt that the fossil deformation model could be applied to the observations in this paper by a sufficiently skilful writer. In other words, although the interpretation offered here is appealing (and very likely correct), it is not the only one possible.

To distinguish between the rival models for the origin of mantle anisotropy, detailed measurements will be needed from the remaining Precambrian cratons elsewhere in Africa and the other continents. Then we should be able to see whether the azimuth of seismic anisotropy is nearly always parallel to the current plate motion, or nearly always parallel to the direction of the original microplate accretion. For now, though, the 'mobilistic' interpretation seems to have overtaken the 'fossil' viewpoint. □

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Action at a distance

Sharon Eden and Howard Cedar

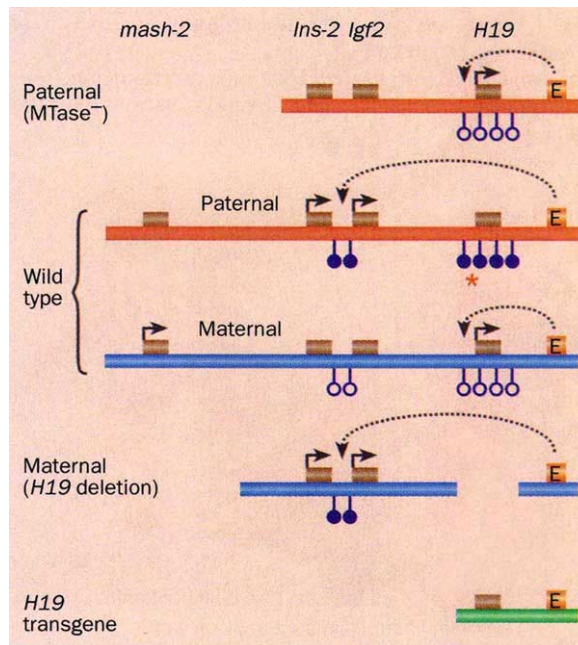
GENOMIC imprinting is a form of developmental gene regulation whereby only one of the parental alleles is expressed. As more examples of imprinted genes are being discovered, it is becoming clear that these sequences are clustered into chromosomal domains, implying that imprinting may be regulated in a regional manner. The precise mechanism is not yet known. But a study by Leighton *et al.*, described on page 34 of this issue¹, for the first time uses a genetic approach to identify some of the *cis*-acting elements involved. In short, the paper shows convincingly that sequences around the *H19* imprinted gene participate in the regulation of two other such genes located over 90 kilobases upstream.

The authors have concentrated on the distal portion of mouse chromosome 7 which carries four known imprinted gene sequences (see figure). Two of them — *H19* and *mash-2*, which respectively encode an untranslated RNA and a helix-loop-helix protein — are expressed exclusively from the maternal allele; and two — the insulin-like growth factor 2 (*Igf2*) gene and the insulin-2 (*Ins-2*) gene in extra-embryonic tissues — are paternally transcribed. To explain this coordinated pattern of allele-specific expression, the authors have previously proposed a simple enhancer-choice model. On the maternal allele, regional *cis*-acting elements preferentially activate *H19*, and are thus unavailable to interact with the distant *Igf2* gene, leaving it in a silent state. On the paternal allele, however, DNA methylation of regulatory sequences flanking *H19* would prevent these contacts and the enhancers would then be free to turn on *Igf2* or *Ins-2*.

One test of this model is to delete the *H19* gene in mice, and so eliminate the enhancer target. This is what Leighton *et al.* have now done. As expected, a paternally derived mutation has no effect on gene expression, but a deletion of *H19* on the maternal allele causes a dramatic developmentally regulated activation of both the *Igf2* and *Ins-2* genes on the same chromosome. In fact, it is this double dose of *Igf2* protein, and not the lack of *H19* expression, which is probably responsible for the 30% or so greater weight of these mice. Further confirmation of the mechanism would necessitate identifying the full complement of *cis*-acting elements that bring about this reciprocal expression pattern. This remains to be done, but there is already good evidence that at least the endoderm-specific enhancers 3' to *H19* can act as regional regulators, because deletion of these sequences from the paternal allele brings about the repression

of *Igf2* in neonatal liver.

Regional control of gene expression may indeed be mediated through long-range regulatory elements, but it is probably the differential methylation of target sequences that brings about allele specificity. The region upstream of and including the *H19* gene, for example, is only methylated on the paternal allele, and experiments using mouse embryos deficient in DNA methyltransferase (MTase) strongly



Model for the regional regulation of imprinting at the wild-type *Igf2/H19* locus. Evidence for the involvement of DNA methylation comes from experiments in methyltransferase-deficient (MTase⁻) mice. The importance of *H19* sequences in the control of imprinting is indicated by the changes in expression which take place in an *H19* knockout mouse, and by experiments with a transgenic construct. The exact locations of all the enhancer sequences (E) which affect expression in this region are not known, but some are located downstream of the *H19* gene. Methylated sites examined in these studies are indicated by open (unmethylated) or closed (methylated) circles. A star marks the presence of a subset of CpG sites, the differential methylation pattern of which is derived from the gametes and maintained through pre-implantation development.

suggest that these same methyl groups are involved in the maintenance of imprinted expression in the region². In these mutant mice, the paternal *H19* allele is abnormally activated, probably because local undermethylation permits the enhancer-mediated *trans*-acting factors to interact with their target sequences. This same global undermethylation also renders the paternal *Igf2* gene transcriptionally silent. This may be a direct result of rechanneling enhancer interactions from *Igf2* to the now unmodified *H19* gene, but it is equally likely that the loss of *Igf2* expression is

due to a local demethylation of putative repressor elements upstream of *Igf2*.

Differential modification patterns are a feature of almost every known imprinted sequence and, once established in the early post-implantation embryo, they are preserved through subsequent cell generations³. So it is likely that DNA modification, by altering protein-DNA interactions in an allele-specific manner, plays a significant role in the maintenance of the imprinted state. But how does each parental chromosome initially acquire its specific methylation profile? This process is presumably mediated either by local 'imprinting boxes', or by a regional-acting

imprinting centre. Both would consist of unique *cis*-acting elements which must already be recognized and marked differentially in the maternal and paternal gametes.

Genomic deletions are generally useful for mapping a wide variety of regulatory elements. But identification of a genuine imprinting centre would also require demonstrating that this sequence, on its own, actually directs gene imprinting *in vivo*. Fortunately, this experiment has already been done by showing that a 14-kilobase *H19* construct (see figure) carries enough information to be consistently imprinted in several independently derived transgenic mouse lines⁴. In the light of this finding, the most likely interpretation of the new *H19* knockout experiment is that this deletion removes the imprinting centre itself, and thereby eliminates the master control element that ultimately regulates allele-specific expression in this region.

This idea is further supported by the observation that the upstream region of the *Igf2* gene on the *H19*-

deleted maternal chromosome actually adopts the methylation pattern of the wild-type paternal allele. Genetic evidence for an imprinting centre has also been found within the locus on human chromosome 15 which is responsible for Prader-Willi and Angelman syndromes; inherited, small and highly localized deletions at this locus lead to dramatic changes in both the methylation and expression patterns of a number of imprinted genes in a 2–4-megabase domain^{5,6}.

It is not yet clear how an imprinting centre might work at the molecular level,

but it has been proposed that untranslated RNA molecules are involved. At least two genes which seem to be involved in imprinting, *H19* and *Xist*, produce untranslated RNA products, and a similar transcript is conspicuously located in the locus for Prader-Willi/Angelman syndromes⁷. The imprinting elements must also carry an allele-specific mark which can be put on in the mature germ cells, but which can also be erased during early gametogenesis in the next generation. Although the nature of this signal is not known, mapping studies have identified small regions

in the *Igf2* receptor (ref. 3) and *Xist* (refs 8, 9) domains which carry allele-specific methyl moieties that are derived directly from the gametes and are maintained during preimplantation development, and it is not surprising that a similar locus has also been identified near the *H19* gene¹⁰. □

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LOCOMOTION

Freeloading women

C. Richard Taylor

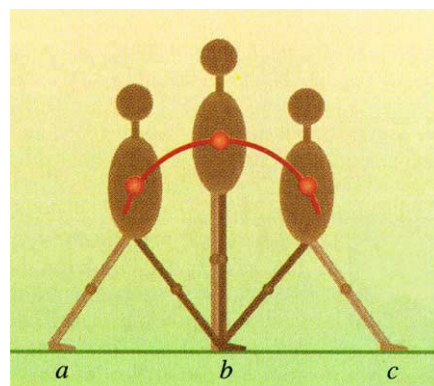
ALMOST ten years ago, a group including N. C. Heglund and myself found that African women of certain tribes can move heavy loads for free — that is, they can carry up to 20% of their body weight on their heads or supported by a strap looped around their foreheads without expending a single additional calorie¹. Anyone else pays a high price for carrying such loads, and tires much sooner. Cost increases in direct proportion to the load for both walking and running in most humans², and a 20% extra load usually increases energy expenditure by 20%.

So what is the women's trick? The energy-conservation mechanism involved has now been analysed by Heglund and other collaborators (page 52 of this issue³). The trick, or at least part of it, is the more efficient use of the body as a pendulum during locomotion.

Energy is conserved during walking, in humans and animals alike, using an inverted pendulum system⁴ (see figure). The body speeds and slows, rises and falls, during each step. It reaches its highest speed (and greatest kinetic energy) at its lowest point, when both feet are on the ground (a in the figure). Then it pole vaults over one leg, slowing as it rises and converting kinetic energy into gravitational potential energy (b). The stored potential energy is converted back into kinetic energy as the body falls and regains its forward speed (c). In a perfect pendulum, the energy transfer would be complete (100%) and no muscular work would be needed to maintain a constant speed. In most humans and animals the exchange is pretty good (about 65%), but it is not

complete and so the muscles must do a little work in each step to supply the lost energy.

Heglund *et al.*³ find that the African women increase the efficiency of transfer as they carry heavier loads, whereas European men and women do not. A force platform mounted in a walkway recorded the gravitational potential energy and the



When walking, everyone uses the pendulum principle to conserve energy. It works by exchanging the body's gravitational and kinetic energy at each step, as discussed in the text, freeing the muscles from some of this work. But the pendulum is usually far from 100% efficient, and muscles consume energy as they replace the mechanical energy lost in the pendulum and as they support the body's weight plus any load. In their paper elsewhere in this issue, Heglund *et al.*³ describe the finding that certain African women lose progressively less energy as they carry heavier loads, until the pendulum exchange is nearly perfect when the load is about 20% of their weight — hence helping to explain why they can carry these loads for free.

forward kinetic energy precisely at each moment, and provided an exact measure of the efficiency of the pendulum. Unfortunately, these experiments do not tell us how efficiency was improved. In any case, relatively more energy is conserved, and relatively less needs to be provided by the muscles.

So part of the mystery is solved — but not quite all of it. The problem is that even if the pendulums reached an efficiency of 100%, muscles would have to be active and generate enough force to support the body's weight (plus any load) regardless of whether they shorten and do work or not. The energy cost of generating this force is what determines the cost of running. It can be predicted remarkably well over the entire range of running speeds in animals ranging in size from mice to horses⁵ simply from the weight that has to be supported and the time available to support it. The cost of generating force also predicts the cost of walking, even in elephants⁶.

There are two ways in which the cost of generating force could be reduced in the African women⁷. The first follows directly from the new finding that they carry out less muscular work. The cost of generating force is lowest when the muscle is isometric and does no work. It increases as the muscle begins to shorten, and is 3–4 times as high at the speed where the shortening muscle works most efficiently. So the cost of generating force decreases as the pendulum becomes more efficient and the work rate of muscles decreases. This leads to a slower shortening velocity and more economical force. The second possibility is a progressively more upright posture with increasing loads, leading to a progressively better mechanical advantage of the limbs against the ground and to less muscle being required to support the body's weight (plus load).

The remaining question is how force is generated more cheaply. This could be answered by combining high-speed film and video analysis with the force platform analysis. Once we have the answer, it should be possible for all of us to emulate these remarkable women and carry heavy loads for free. □

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Rebirth of a sea-floor vent

Wayne C. Shanks

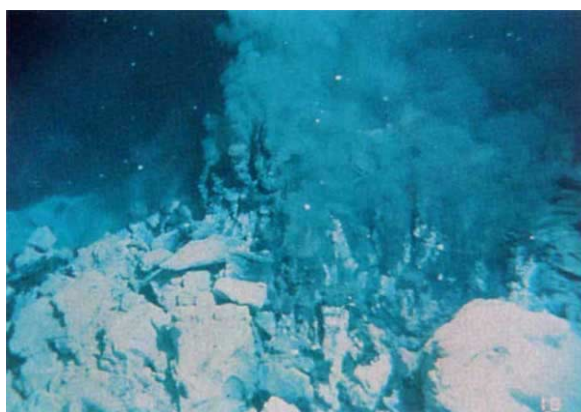
In 1979, the Earth science community was stunned by the discovery of black smokers — deep-sea vents which, as the name suggests, emit sooty jets of fluid — on the crest of the East Pacific Rise. The initial discoveries were followed in rapid succession by similar finds at numerous other sites on the globe-encircling system of mid-ocean ridges. About 27 vent localities are now known¹, but at only one have researchers been lucky enough to catch a smoker almost at the moment of its birth. On page 47 of this issue, Von Damm and co-workers² describe what happened next.

Most vents consist of localized jets of sulphide-mineral-laden fluids that issue from very young volcanic crust, and often form self-made chimneys of metallic sulphide and sulphate minerals, sometimes several tens of metres high. Around the vents are found exotic colonies of previously undiscovered macro- and microfauna specially adapted for chemosynthesis using hydrogen sulphide from the vent fluids as an energy source. The discoveries revolutionized thinking about the role of the oceanic spreading centres in the heat budget of the Earth and geochemical cycles of the elements. For example, calculations indicate that the entire world ocean circulates through the ridge crests every million years, and that 25 per cent of the total heat loss from the Earth is by marine hydrothermal circulation¹. The vent communities also cast a different light on the ecological importance of chemosynthetic life forms — and their relationship to the development of life on Earth.

An enigmatic aspect of the vents studied in the 1980s has been that the composition of the hot vent fluids seemed remarkably stable. Over periods of 6 years and longer, the fluid compositions from individual vents show little change, despite measured changes in temperature and flow characteristics³. This chemical stability has been attributed to thermodynamic equilibrium with rocks. But the vent sites between 9° 45' and 9° 52' N on the East Pacific Rise are different. There, a volcanic eruption destroyed the existing vent system in 1991. Only weeks later, as the system was starting to re-establish itself, the submersible *Alvin* was in the area and able to take the first of a series of measurements. Sampling and analysis of vent fluids, especially from the 'A' vent at

9° 46.5' N discussed by Von Damm *et al.* (see photo), show dramatic deviations from static chemical and physical conditions over the initial three years.

What do these observations tell us about the geological, physical, chemical and biological processes that occur in a mid-ocean-ridge vent system? The most obvious geological and physical results of the 1991 eruption provide a framework. The eruption affected a narrow, axial area of the ridge crest for a length of about 12 km (ref. 4). Many of the post-eruptive



Hot stuff — vapour at 403 °C venting from basalt rubble at 9° 46.5' N on the East Pacific Rise, following the April 1991 eruption. White material is bacteria.

hydrothermal vents returned to sites that had previously been well-developed vents with associated mature biological communities (tubeworms, clams, mussels, crabs and so on)⁵. Substantial temperature changes, both in the short term (days to weeks) and long term (months to years) have been documented for individual vents² — including evidence for boiling, with fluids in the vapour phase.

Taken together, these observations indicate that the cause was injection of basalt to form a dyke, with accompanying sea-floor lava eruption in some areas. The length of ridge crest affected is almost exactly the same as the strike length of rift-related dykes in Iceland⁶. Re-occupation of previous vent sites by new post-eruptive vent fields indicates that there is long-term control, either by some sort of cross-structure or preferential use of previously established hydrothermal fluid conduits (perhaps because they are pre-heated). Temperature variability probably stems from permeability changes related to dyke emplacement and heating of the surrounding rocks, followed by a rapid cooling of thin dyke injections.

The most striking geochemical variations observed by Von Damm *et al.*² are those of chloride and SiO₂ in the vent

fluids; both decreased dramatically as the temperature increased (from 389 to 403 °C) over a two-week period at the A vent in April 1991. These changes clearly are related to chemical processes in the shallow subsurface, probably due to interaction with a shallow dyke. Salinity deviations from seawater values have been recognized from the earliest geochemical investigations of the warm springs on the Galápagos Ridge⁷. Subsequent sampling at many sites has shown a salinity range from 30 to 200 per cent of seawater values, but the cause of such variations has been hotly debated^{8–11}. The observations by Von Damm *et al.* extend this range to nearly pure vapour with a salinity of about 6 per cent that of sea water and, for the first time, unequivocally demonstrate active phase-separation (between liquid and vapour) at normal mid-ocean-ridge depths on the sea floor, although it has been seen at abnormally shallow depths on Axial seamount¹².

The sharp decrease in temperature of the A vent to 332 °C in 1992, while salinity remained at less than half that of sea water, confirms another unexpected phenomenon — segregation and storage of phase-separated fluids in the subsurface for later venting when active phase-separation is no longer occurring.

Curiously, the 1991 observation shows that SiO₂ concentration is controlled by chemical equilibrium with quartz. Chemical equilibration occurs after phase separation in the rapidly ascending fluids, even though the phases separate at depths of less than 200 m below the sea floor. Stable isotope studies of hydrogen, oxygen and sulphur in these vents similarly show a preponderance of effects due to reactions between water and rock¹³, with hydrogen isotope evidence of a small

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magmatic water contribution in the initial weeks following the eruption.

Finally, an additional surprise in the days immediately following the 1991 eruption was the extraordinary abundance of bacteria in the water column and in thick mats on the sea floor, possibly supported by extremely high concentrations of H_2S in the vapour-rich vents. Yet, by

1993, the thick accumulations of bacteria were gone, and macrofaunal assemblages had redeveloped¹⁴. Clearly, geophysicists, geochemists and biologists need to work together closely to understand the effects of discrete eruptive and tectonic events on global heat and geochemical budgets, and on sea-floor biodiversity. Interdisciplinary efforts aimed

at rapid response to volcanic eruptions, time-series monitoring and setting up ocean-floor observatories are paying big dividends in understanding these dynamic and complex processes^{4,9,15-17}. □

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OBITUARY

Harold M. Weintraub (1945–95)

I FIRST met Harold Weintraub, who died on 28 March, in 1971. As a 25-year-old MD/PhD student working with Howard Holtzer at the University of Pennsylvania, he came to visit my new laboratory in Princeton to investigate taking a post-doctoral position with me. He had been studying how cells make the decision to differentiate and was intrigued by hints that DNA replication might facilitate the permanent changes in gene expression that determine cell fate. Hal wisely decided to do his post-doctoral work with Sydney Brenner and Francis Crick in England, but he had made such a powerful impression that we quickly recruited him back to Princeton as an assistant professor in 1973.

Hal Weintraub was to remain obsessed with the question of how cells change from one state of determination to another during embryonic development for his entire scientific career. Initially he focused on the intriguing (and still uncertain) idea that the histones, and the chromatin structures that they form, play a central part in the uniquely stable changes in gene expression that occur as eukaryotic cells differentiate. A fearless experimentalist, he probed the structure of chromatin with all of the tools he could imagine — chemicals, proteases and nucleases, in all combinations. With his post-doctoral fellow, Mark Groudine (later a long-term colleague and close friend), he discovered that the globin gene becomes 'open', as judged by its susceptibility to digestion by pancreatic DNase I, long before it is actually expressed in immature red blood cells. The mechanisms involved are fascinating and are still under investigation in many laboratories.

Hal seemed to be born to do science. I remember walking into his empty shell of a laboratory the week after he arrived in Princeton: somehow he had already managed to borrow enough glassware and reagents to begin a major experiment. Highly innovative, his interest and energies were infectious. Soon he had most of the other assistant professors at Prince-

ton (including myself) thinking about how embryos develop. Through journal clubs, evening special topics courses and seminars, we worked ourselves into a frenzy of excitement thinking about the molecular mechanisms that cells might have developed to make multicellular life possible. Hal's influence probably explains why Marc Kirschner, Abe Worcel, Uli Laemmli and I — all of whom arrived

came two marvellous discoveries. First there was the demonstration that the introduction of antisense RNA molecules into cells could shut down the expression of specific genes, providing a powerful new tool for the dissection of cell function. A few years later, a novel search strategy for a master gene regulatory protein involved in muscle development culminated in the discovery of MyoD, a DNA-binding protein that can convert a variety of other cell types into myoblasts, as well as boost its own synthesis to provide the type of cell memory required to explain the stability of cell determination events. The discovery of MyoD altered the way we think about the changes in gene expression that occur during embryonic development, the very same problem that had obsessed Hal since his days as a graduate student.

Everyone who knew Hal will recognize that what I have written thus far is inadequate to describe his quality. He inspired many young scientists. Perhaps most remarkably, his whole life consisted of a search for novel and interesting ideas, and he derived at least as much joy from those of others as he did from exploring his own. My last visit with him was typical: although he had been diagnosed with an incurable brain tumour in September 1994, Hal continued to concentrate on science until his death. During a three-hour breakfast in December, we marvelled at the complexity of cell signalling networks, at the way that cells have made microswitches out of proteins and at the whole range of ingenious mechanisms that make life possible. His face was aglow with the warm smile that typified this unassuming man; his love for science seemingly had allowed him to forget his impending mortality.

Scientists like Harold Weintraub are rare, and his death leaves an empty space in our community. Bruce Alberts

Bruce Alberts is currently President of the National Academy of Sciences, 2101 Constitution Avenue NW, Washington DC 20148, USA.

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Hal Weintraub — love for science.

at Princeton with little or no background for this problem — started working on some aspect of the molecular biology of embryonic development within a few years.

Throughout his career, Hal kept his focus on the central biological problem of how cells in embryos change their patterns of gene expression in highly organized ways. He left Princeton in 1978 and moved to what was then a relatively obscure research laboratory where he could spend more time doing experiments himself. Attracted by his presence, many of the country's most promising young scientists would soon join him there; as a result, the Fred Hutchinson Cancer Research Center in Seattle quickly became one of the world's most exciting biological research centres. Its tremendously successful coalition of small, highly interactive laboratories is based on Hal's insistence that laboratory leaders should have the time to do experiments themselves. In this and other ways, he produced a model collegial environment.

From this laboratory there eventually

Big flies have bigger cells

R. McNeill Alexander

It is generally assumed that related animals of different sizes have similar-sized cells. The red blood cells of mammals are often quoted as an example: their diameters are about 8 μm , both in shrews and in humpback whales¹.

However, R. D. Stevenson and his colleagues² have now shown that bigger fruitflies have markedly bigger cells. They studied the *Drosophila* species of Hawaii, which range in length from a smallish 2 mm to a giant 8 mm. Sensibly, they chose to measure cells whose sizes are apparent from the fly's external morphology. They inferred the sizes of cells in the wings from the spacing of hairs (there is one hair for each cell); the sizes of photoreceptors from the sizes of the facets of the eye; and those of cells in the feet from the spacing of bristles (alternate cells carry a bristle). In each case, the 2 mm flies had (roughly) 15 μm cells and the 8 mm flies had 25 μm cells.

There are limits to the range of feasible sizes for a cell. It must be large enough to contain the genome (and salamanders with exceptionally large genomes have larger brain cells than others with smaller genomes³), but not so large that the nucleus cannot control it.

Knowledge of cell size is critically important for understanding the allometry of some organs. Consider, for example, the gills of fishes, whose lamellae consist of two sheets of epithelial cells linked by pillar cells. Blood flows through the space between the two sheets, which is just wide enough to allow red cells to pass. As for other groups of animals, the metabolic rates of fishes are roughly proportional to (body mass)^{0.75} (ref. 4). How can the gills of fishes of different sizes take up oxygen at the appropriate rates?

Suppose first that fishes of different sizes are geometrically similar, with cell diameters (and gill filament thicknesses and spacing) proportional to body length, or to (body mass)^{0.33}. Gill area will be proportional to length squared, or to (body mass)^{0.67}. Oxygen will diffuse into the blood at a rate proportional to gill area and inversely proportional to diffusion distance — that is, at a rate proportional to (mass)^{0.67}/(mass)^{0.33} = (mass)^{0.33}. If metabolic rate is proportional to (mass)^{0.75}, then big fish will be short of oxygen.

Now suppose instead that different-sized fishes have gills built from cells of equal size. The thickness of the gill lamellae will be constant, and we can expect their spacing also to be constant so that the ratio of the pressure needed to pump water over the gills, to that needed to pump blood through them, will be con-

stant. In that case gill area will be proportional to the volume of the gills, or to body mass, and diffusion distance will be constant. Oxygen will diffuse in at a rate proportional to (body mass)^{1.00}/(body mass)⁰, that is to body mass. With this set of assumptions it is the small fish that will be in trouble, if metabolic rate is proportional to (body mass)^{0.75}.

To get diffusion rates proportional to (body mass)^{0.75}, cell size and the dimensions of the lamellae should be proportional to (body mass)^{0.125}. Observed exponents are less than this, 0.02 and 0.09 in ontogenetic series for two species⁵. Stevenson *et al.*² relate fruitfly cell diameters to the length rather than the mass of the body, but from their data it can be estimated that cell diameters are proportional to body mass with exponents between 0.11 and 0.17.

Now we realize that cell size may vary allometrically, we may expect it to be optimized. Barlow's classic argument⁶ describes what this might involve in the compound eyes of insects. These must have small facets, to be capable of seeing fine detail. But excessively small facets will be less good at resolving detail, because diffraction makes them less directionally selective. The best compromise is to have facet (and receptor cell) diameters proportional to the square root of eye diameter. Barlow found that the eyes of bees and wasps scaled almost as predicted. Stevenson *et al.*² find that facet diameter in *Drosophila* eyes is proportional to (eye diameter)^{0.68 ± 0.21}.

Attempts to predict optimum allometric relationships are plagued by the problem of excessive numbers of variables. Geometric similarity and elastic similarity⁷ are convenient simplifying assumptions in some cases, but are often contravened. Constancy of cell size seemed highly attractive, as an assumption that might be widely applicable. But it is now clear that it should not be made blindly. □

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The social market

LAST week Daedalus proposed that students should be supported, not by grants or loans, but by shares. He is now taking this scheme to its ultimate conclusion, combining capitalism and communitarianism. Every human activity, he says, should be directed not by ideologies but by market forces, and funded not by taxation but by shares.

A newborn baby, for example, can be regarded as a promising technical project in a very early stage. It will make a profit only after years of development. At present, the parents may back it with their own resources, or depend on State benefits. Their quality of management can vary wildly, and may be very poor — as with a deprived single mother.

But suppose such a mother, instead of relying on the State, has to fund her offspring by issuing share capital in it. To attract investors, she will have to bring it up as best she can, keep it out of crime and not burden her portfolio with further potential loss-makers. If she fails, the child's share price will decline, and it may be taken over by another mother or a specialized institution which can manage it better.

When the child finally grows up, he will pay dividends to his shareholders. This need not be much of a burden. Nearly all State spending these days goes on benefits and entitlements; once these are abolished, income tax can go as well, with the purely personal dividend in its place. The sound financial discipline which constrained the parents will now devolve upon the offspring. If he seems not to be fulfilling his potential, his share price will suffer, and he will find it hard to raise capital for projects of his own. If he goes seriously to the bad, he will probably be taken over and turned round by some firm specializing in rescuing bad risks such as dead-end kids, drifters and mid-life crisis. An efficient market solution will supersede clumsy, costly social services.

Even in old age, he need not suffer. During his productive lifetime, he will himself have bought stock in the next generation of children. Their dividends will be his pension, and he will trade mainly as a holding company.

At every stage in life, his shareholders will be there, providing support and discipline: backers, managers and godfathers combined. The incompetent State will be rolled right back, replaced by a collective social market which will grow ever shrewder and more efficient as it gains experience. And our modern, dangerously diverse society will be unified and harmonized, as each member acquires multiple financial interests in all the others. David Jones

Far-red sensitivity of dragon fish

SIR — Most deep-sea fishes have visual pigments maximally sensitive ($\lambda_{\max}$ 470–490 nm)¹ to the blue light that most easily penetrates clear oceanic waters², providing one of the best examples of visual pigment adaptation to the environment. Yet the Sun is not the only source of visible radiation: many deep-sea animals produce their own light. Generally, this bioluminescence matches the downwelling light³, but three genera of mesopelagic stomiid dragon fishes (*Malacosteus*, *Pachystomias* and *Aristostomias*) have, in addition to blue-emitting light organs, suborbital photophores that produce far-red light^{4–6}.

These animals also have in their retinae two visual pigments that are red-shifted compared with those of other deep-sea fish, absorbing maximally around 520 and 550 nm^{1,7–9}. However, the spectral overlap between these long-wave-sensitive visual pigments and the far-red bioluminescence (peaking around 700 nm) is very limited. The visibility of these bioluminescent signals would be significantly enhanced by the possession of visual pigments absorbing at longer wavelengths, such as are found in the cones of many shallow fresh-water fishes. Tantalizingly, a preliminary report by Denton *et al.*⁴ suggested that one stomiid, *Pachystomias microdon*, might indeed have a visual pigment with a $\lambda_{\max}$ at 575 nm, although later studies failed to confirm this suggestion^{1,8}.

We caught three specimens of *Aristostomias tittmanni* using a rectangular mid-water trawl with a closing cod end, in the tropical region of the northeastern Atlantic (catch sites within the area 17°58'N to 21°24'N; 17°39'W to 19°19'W) during RRS *Discovery* cruise 204. Immediately after net hauls were brought on deck, animals, all of which were dead, were transferred to light-tight containers and kept in darkness floating in iced sea water. In a darkroom, and working under extremely dim red light, we removed retinae from hemisected eyes and either used them immediately or preserved them by freezing or aldehyde treatment¹⁰, for visual pigment spectrophotometry ashore. We measured visual pigment absorption spectra by two well established

methods: microspectrophotometry, which yields absorption spectra of individual retinal photoreceptor outer segments¹¹; and spectrophotometry of detergent extracts of retinae combined with partial bleaching with long-wave monochromatic light to identify the component visual pigments in

A, Absorbance spectra of a whole-mount preparation of a fresh piece of *Aristostomias tittmanni* retina following sequential bleaches. In order of decreasing absorbance at 550 nm these are: initial measurement 10 min after the addition of hydroxylamine (a); after 10 min exposure to 699 nm light (b); 60 min 699 nm (c); 10 min 666 nm (d); 30 min 666 nm (e); 60 min 666 nm (f); 60 min 666 nm (g); 60 min 666 nm (h); 77 s 609 nm (i); 77 s 609 nm (j); 6 min 521 nm (k). A small piece of retina, approximately 4 mm in diameter, was sandwiched between stainless steel mesh, bathed in PIPES-buffered saline¹⁰ containing 50 mM NH_2OH , and held in a cuvette mounted in the beam of a Shimadzu UV-2101PC spectrophotometer in front of an integrating sphere.

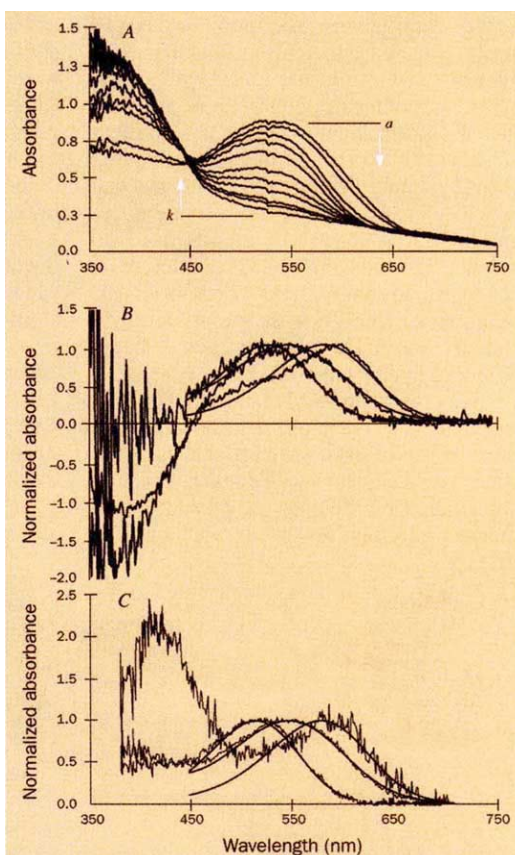
B, Normalized difference spectra constructed using the curves h–j, c–e and a–c in A. Best-fitting visual-pigment templates¹⁷ are shown (smooth lines) for P531₁, P550₂ and P586₁. **C**, Normalized absorbance spectra from microspectrophotometry measurements¹¹ of the three types of rods found in the glutaraldehyde-treated¹⁰ retina. Averaged raw data, from artefact-free scans¹¹, are shown with best-fitting visual pigment templates. The $\lambda_{\max}$ values, absorbances at the $\lambda_{\max}$, and the number of scans included in each average are: P518₁ (0.027; $n=18$); P550₂ (0.022; $n=42$); P581₁ (0.014; $n=2$). Rod outer-segment diameters and lengths (mean $\pm$ s.d.) were $2.4 \pm 0.5 \times 14.8 \pm 5.1$, $2.1 \pm 0.21 \times 15.4 \pm 3.8 \mu\text{m}$, and $< 1 \times 7 \mu\text{m}$, respectively.

Measurements were from individual rod outer segments except in the case of the long-wave rods which were measured as a tissue patch of several cells. No hydroxylamine was added to the bathing medium and a high short-wave absorption ($\lambda_{\max} \sim 419 \text{ nm}$) was found in the long-wave rods, suggesting that this pigment had bleached before measurement, was unstable in aldehyde-treated tissue¹⁰, or that the outer segments contained a short-wave-absorbing photostable pigment.

the extract¹⁰. We also measured the absorption spectra of small pieces of intact retinae using a novel retinal whole-mount technique¹⁰ together with partial bleaching.

In general agreement with previous data^{7,8}, detergent extracts revealed the presence of two visual pigments: a

rhodopsin and a porphyropsin based on the same opsin with $\lambda_{\max}$ values of 523 and 551 nm (see table). However, spectrophotometry of fresh retinal whole mounts from the same animals revealed an additional long-wave-absorbing rhodopsin with a $\lambda_{\max}$ between 586 and 590 nm (see figure and table). Microspectrophotometry of a small part of a single retina also revealed three visual pigments (P518₁, P550₂ and P581₁)



VISUAL PIGMENT $\lambda_{\max}$ VALUES OF <i>ARISTOSTOMIAS</i> SPP. MEASURED BY THREE DIFFERENT METHODS				
Species	Microspectrophotometry	Extract	Whole-mount	Ref.
	$\lambda_{\max}$ (nm)	$\lambda_{\max}$ (nm)	$\lambda_{\max}$ (nm)	
<i>A. scintillans</i>		526 ₁ , 551 ₂		7
<i>A. grimaldii</i>		517 ₁ , 552 ₂		8
<i>A. tittmanni</i>	518 ₁ , 550 ₂ , 581 ₁			Present
		523 ₁ , 551 ₂		(aldehyde treated)
			531 ₁ , 550 ₂ , 586 ₁	Present (fresh)
			531 ₁ , 546 ₂ , 590 ₁	Present (fresh)
			527 ₁ , 546 ₂	Present (frozen)

Although three different species of *Aristostomias* are listed above, it is difficult to differentiate between these species with absolute certainty. Subscripts indicate rhodopsin (₁) or porphyropsin (₂) visual pigments.

located in three types of rod-like photoreceptors (C in the figure; table). Interestingly, whole mounts of previously frozen material showed only the two short-wave visual pigments (table). The most likely explanations for the presence of the long-wave rhodopsin in the microspectrophotometry and fresh whole-mount data, and its absence in extracts and previously frozen material, are that this pigment is unstable in detergent, cannot be easily extracted, or is destroyed by freezing. Given the presence of both rhodopsins and porphyropsins in the retina of *Aristostomias*, it is perhaps surprising that we did not find the porphyropsin analogue of the long-wave rhodopsin (P658₂).

Taking available measurements¹² of photophore emissions from the closely related fish *Malacosteus niger* as a basis for calculations, we estimate that stomiids produce about 4×10^9 and 4×10^{10} pho-

tons per second per steradian from the red and blue photophores, respectively. Given the known emission spectra of *Aristostomias*⁵ and the spectral beam attenuation coefficients of the clearest ocean waters², it is possible to calculate the flux of light reaching a target from a point source photophore¹³ as well as that being reflected back to the emitting fish. Using measurements of the spectral transmission of *Aristostomias* ocular lenses (unpublished observation) and pupil areas (10 mm²), with the information that *Aristostomias* has a bilayered rod retina with a total outer-segment path length of 30 μ m (ref. 8) and outer-segment-specific absorbances of $1.2 \times 10^{-2} \mu\text{m}^{-1}$, the total retinal photon capture of the emitting animal can be calculated. There are no measurements of absolute sensitivities of fishes to point sources of light, but for the detection of a point source humans need about 200 photons per s entering the pupil¹⁴, equivalent to about 30 photons per s being absorbed by the retinal rods¹⁵.

Using this value, and assuming that *Aristostomias* has a random distribution of its three types of rod, we can calculate the maximum distances at which targets will be detectable by reflection, or at which conspecifics could be observed. Such calculations

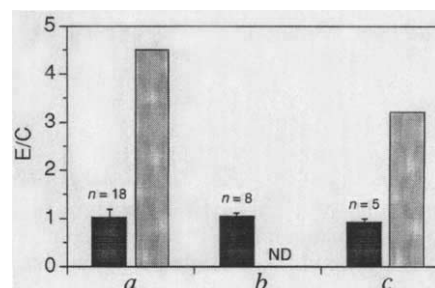
show that the detection of conspecifics' emissions would be possible, in the clearest ocean waters, to 37.1 and 2.0 m for the blue and red photophores, respectively. If the light is reflected from a target of 95% reflection, these distances are 7.2 and 1.3 m, respectively, and 4.3 and 0.8 m for reflections of 10%. *Aristostomias* is a piscivore (T. Sutton, personal communication) specializing in lantern fish, which have a single-layered retina with rods about 15 μ m long and λ_{max} values around 490 nm. These myctophids should be able to see the blue photophore of *Aristostomias* to distances of 36 m but will only see the red photophore at less than 10 mm. Thus, the red photophore of *Aristostomias* could be used both for private, intraspecific communication, invisible to most deep-sea animals, and for covert illumination of prey at distances about ten times greater than the range of lateral line senses¹⁶.

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Changes in steady-state mRNA levels in HL60 cells exposed to a 60-Hz, 5.7- μ T magnetic field for 20 min. Gene expression in exposed relative to sham-exposed cells was determined using mRNA levels that had been normalized to a β_2 -microglobulin internal standard (solid bars). Standard deviations for each data set are shown along with the number of independent experiments performed (*n*). For comparison, previously published data⁶ from identical exposures is shown (shaded bars). *a*, *MYC* expression in exposed compared with sham cells. *b*, *MYC* expression in a sham-sham exposure. *c*, β -Actin expression in exposed compared with sham cells. ND, Not determined in the previous work. E/C, Ratio of expression in exposed cells to that in sham-exposed controls.

showing increased expression of the proto-oncogene *MYC* in a human leukaemic cell line, HL60, has drawn considerable attention⁶⁻¹⁰. Yet in these studies, the quantification of *MYC* expression was not normalized to any internal standard, nor were any positive controls used to demonstrate the sensitivity of the assays. The fact that all the diverse genes tested — *MYC*, β -actin and histone H2B — showed the same apparent response went unquestioned.

We have undertaken an extensive series of experiments, which will be published in full elsewhere, to validate these claims. Using ribonuclease protection assays with simultaneous quantification of either *MYC* or β -actin and a β_2 -microglobulin internal standard, we found no evidence for EMF-induced alteration of gene expression under the conditions previously reported to show the maximum effect for power frequency fields (see figure). Positive controls in each assay verified that small changes of 10% would have been detectable.

Even though our data question the entire notion that changes in gene expression can be induced by magnetic fields, the results do not necessarily imply that field effects on gene expression or other cellular processes would not occur under different conditions. They do, however, demonstrate the importance of essential controls in this research. Critical evaluation of much of the bioelectromagnetics literature uncovers similar shortcomings. Thus, it is not necessary to invoke magnetite as an explanation for these effects, which in fact may not exist.

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Cancer risk and electromagnetic fields

SIR — The issue of health hazards associated with weak, extremely low-frequency electromagnetic fields (EMFs) as produced by power lines and household appliances continues to be debated. Epidemiological studies investigating the link between EMFs and increased risk of certain cancers have not clarified the issue¹⁻⁴. Thus, researchers have looked to laboratory studies on EMF effects to determine whether there is biological plausibility for increased cancer risk. Unfortunately, those laboratory studies themselves remain equivocal.

Kobayashi et al.⁵ have now raised an important concern, suggesting that magnetite particles found in cell culture media and typical laboratory plasticware may be responsible for the apparent biological effects of weak EMF fields. Investigators reporting these effects have not controlled for such particles.

Nevertheless, we do not consider it necessary to invoke this alternative explanation for much of the bioelectromagnetics literature. Before considering the role of potential confounders to explain the effects of EMFs on biological systems, it is of foremost importance to determine whether there is indeed an effect to explain — that is, do the data reported support the conclusions drawn by the investigators?

Almost all the reported biological effects of EMFs have been at the limit of resolution for the assays used. Given this situation, one would expect that every useful control would be included. But, on the contrary, even the most highly cited reports of EMF biological effects (those on gene expression effects; for example, refs 6, 7) lack essential controls.

Because the potential link between EMF exposure and leukaemia has been a primary concern, the data purportedly

TEST OF EFFECT OF ELECTROMAGNETIC FIELDS ON CELLS

Expt series	Preincubation time (h)	Exposure time (min)	EMF ($\mu\text{T r.m.s.}$)	MYC	n	GAPDH	n	β -actin mRNA	n
1	1	20	5.7	1.10 (0.89, 1.36)	5	—	—	1.15 (1.08, 1.22)	5
2	1	20	5.7	1.06 (0.94, 1.19)	5	—	—	1.06 (0.86, 1.30)	5
3	1	20	5.7	0.93 (0.86, 1.00)	12	0.95 (0.81, 1.12)	12	0.93 (0.81, 1.07)	7
4	1	60	5.7	0.93 (0.69, 1.25)	5	0.87 (0.77, 1.00)	5	0.85 (0.71, 1.03)	5
5	12	20	0.57	1.01 (0.92, 1.11)	5	0.92 (0.79, 1.08)	5	0.89 (0.72, 1.09)	5
6	12	20	5.7	0.98 (0.87, 1.12)	10	1.08 (0.95, 1.23)	10	1.11 (0.97, 1.26)	10
7	12	20	5.7	1.10 (0.98, 1.24)	5	0.99 (0.63, 1.57)	3	1.00 (0.90, 1.11)	7
8	12	20	0.1	1.04 (0.87, 1.25)	4	1.10 (0.98, 1.24)	4	—	—
9	8 or 12	20	1.0	0.97 (0.88, 1.07)	9	1.03 (0.93, 1.13)	5	—	—
10	8 or 12	20	10	1.05 (0.96, 1.15)	10	1.05 (0.93, 1.18)	7	—	—
11	12	20	100	0.81 (0.56, 1.17)	4	0.97 (0.84, 1.12)	3	—	—
3–11				0.98 (0.93, 1.03)	64	1.00 (0.94, 1.05)	54	0.98 (0.91, 1.05)	34

Experiments 1–7: Two sets of coils (applied a.c. field horizontal) constructed to the specifications of ref. 20 were located one on either side of a vertical mu-metal baffle within a mu-metal box in a water-jacketed incubator. Suspensions of HL60 cells (15 ml, $0.8\text{--}1.02106 \text{ ml}^{21}$ in RPMI 1640 medium containing 10% FCS) in T25 culture flasks were located in the centre of the coils. Experiments 8–11: six Helmholtz coil sets (applied a.c. field vertical), arranged with three in an equilateral triangle on either side of the vertical mu-metal baffle, were used. Cell suspensions (5 ml) as above were placed in the centre of the coils in 35-mm Petri dishes. Temperature ($37 \pm 0.05^\circ\text{C}$), CO_2 ($5.0 \pm 0.05\%$) and magnetic field (residual geomagnetic field $<1 \mu\text{T}$, a.c. background $<5 \text{ nT r.m.s.}$) inside the mu-metal box were logged every minute. No increase in background magnetic fields could be detected at the sham exposure coils at any of the field strengths used. The specific gene mRNAs from EMF-field-exposed (E) and control (C) cells were quantified from northern blots by phosphorimager and E/C ratios are given with 95% confidence limits. Expt 1: MYC and β -actin E/C ratios calculated without normalization for the amount of RNA in each sample; expt 2: the amounts of MYC and β -actin mRNA were normalized to the house-keeping gene, GAPDH; expts 3–11: ^{14}C rRNA labelling used for normalization; bottom row: means for experiments using ^{14}C rRNA normalization. To label cellular RNA with ^{14}C -uridine, cells were either incubated ($0.5 \mu\text{Ci}$ per 30 ml) throughout the 8- or 12-h preincubation in the field exposure incubator before field exposure, or were prelabelled for 16 h, concentrated by centrifugation and re-suspended in fresh complete culture medium with ^{14}C -uridine before placing in the field exposure incubator for 1 h preincubation before field exposure. A full technical account of these experiments will be described elsewhere.

SIR — Epidemiological studies have shown weak correlations between exposure to extremely low-frequency electromagnetic fields (EMFs) and cancers, particularly childhood leukaemias^{11–13}. These observations prompted many *in vitro* cellular studies in which effects of EMFs were reported. However, no reported response has been widely and independently replicated, which is essential for critical evaluation. Key reports by Goodman and Henderson were the activation of proto-oncogenes in human leukaemic (HL60) and other cells^{8,14–16}, because of the essential role of proto-oncogenes in proliferation. EMFs increased the amounts of messenger RNA for MYC and β -actin in HL60 cells by 2–4-fold after 20 min exposure to a 60-Hz horizontal sinusoidal field (5.7 mT r.m.s.)^{8,14}, although smaller effects for the same genes (mean 1.3-fold) were reported subsequently^{15,16}. We have attempted a systematic replication of this effect.

HL60 cells were exposed to EMFs (expt 1 in table above) under very similar

conditions to those described^{8,14}, except that coil sets for exposed and control cells were switched randomly between experiments so that the experiments were performed blind, and mRNAs were quantified from northern blots rather than dot blots. The ratio of MYC mRNA from exposed and control cells was 1.10, with large confidence limits of 0.89 and 1.36 due to the absence of normalization by direct measurement of the amounts of RNA in each sample.

In further experiments, we observed no EMF effect when MYC and β -actin mRNAs were normalized to the house-keeping gene, GAPDH (expt 2 in table), or by labelling the RNA with ^{14}C -uridine (expts 3–11), although the confidence limits were substantially reduced. In all experiments, TPA (phorbol 12-myristate 13-acetate) was used as a positive control for activation of the MYC gene. The ratio of MYC mRNA in TPA-treated cells to control cells was 2.18 (2.01, 2.36; $n=9$) and therefore field effects of the magnitude reported should be readily detectable.

Unsatisfactory features of previous procedures^{8,14} are that the cells were equilibrated for only 1 h before the field was applied and after exposure were placed on ice for 10 min at ambient EMFs before centrifugation and RNA preparation. We therefore performed experiments in which cells were maintained in the exposure incubator for 12 h before the field was applied and RNA was prepared immediately after removing the cells from the incubator. No effect on MYC or β -actin expression was observed (expts 5–7 in table). Experiments in which the cells were lysed *in situ* before they were removed from the incubator showed no effect on MYC expression (expts 8–11). Summation of the data for experiments 3–11 gave a mean ratio for MYC mRNA of 0.98 (0.93, 1.03).

It is very unlikely that a MYC gene response depends on the subculture of HL60 cells used, as Goodman and Henderson have reported responses to EMFs in seven different types of cells^{8,14–19}, with no reports of cell types insensitive to EMFs. It is therefore likely that either there is an elusive requirement for an EMF gene response yet to be defined or that there was a systematic error in the experiments showing a positive response. The data do not eliminate the possibility of very small EMF effects on gene expression in HL60 cells below the levels defined here.

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PUMPing plants

SIR — Flowering and fruit ripening are triggered by a burst of respiration and heat production possibly related to a mitochondrial, cyanide-resistant, uncoupled electron transport pathway¹. In mammals, transient thermogenesis is linked to a mitochondrial uncoupling protein² (UcP), believed to be a late evolutionary acquisition³. A detailed analysis of respiratory control of plant mitochondria led us to propose the existence of an UcP-like factor in plants⁴.

Addition of ATP to potato mitochondria produced a 52% decrease in the rate of state-4 respiration and an increase of about 15 mV in the value of the membrane potential ($\Delta\Psi$) (Fig. 1a). The ATP-induced changes in respiration rate and $\Delta\Psi$, compatible with increased mitochondrial coupling, were unaffected by

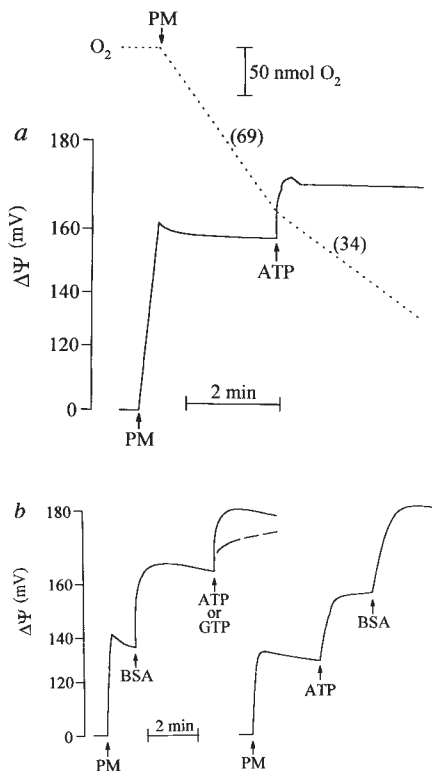


FIG. 1 Effect of ATP, GTP and BSA on transmembrane potential and resting respiratory rate of potato tuber mitochondria (PM). *a*, PM (1 mg protein ml⁻¹) were added to a solution of 300 mM mannitol, 20 mM KCl, 10 mM HEPES buffer pH 7.2, 3 mM tetraphenylphosphonium (TPP⁺), 5 mM succinate and 0.1% BSA. ATP (0.1 mM) was added as indicated. The numbers in parentheses are rates of oxygen uptake in nmol per min per mg protein. Oxygen was measured with a Clark electrode in a 1.0-ml magnetically stirred glass chamber. Membrane potential was estimated by measuring extramitochondrial TPP⁺ as described by Kamo *et al.*⁷. *b*, PM, isolated in the absence of BSA, were incubated in a medium similar to that in *a*, lacking BSA. BSA (Sigma, fatty acid free) (1%), 0.1 mM ATP or GTP were added as indicated. Results are representative of 5 separate experiments.

oligomycin (2 $\mu\text{g ml}^{-1}$) or carboxyatractylide (20 mM) (not shown). The membrane potential of isolated potato mitochondria, isolated and incubated without bovine serum albumin (BSA), increased additively by additions of BSA and ATP (Fig. 1b), whereas GTP caused a smaller but significant increase (Fig. 1b, dashed line). These results suggested the presence of an H⁺ conductance in potato mitochondria similar to that produced by UcP.

We isolated a protein of relative molecular mass 32,000 (32K) from potato mitochondria and purified it following a procedure used to prepare UcP. We call this protein plant uncoupling mitochondrial protein (PUMP). A common property of the mitochondrial P_i carrier, the ATP/ADP translocator and UcP is that these proteins are not retained by hydroxylapatite and at room temperature only UcP is obtained. Polyacrylamide gel electrophoresis revealed the presence of a 32K band both in the brown fat and in plant mitochondrial isolates (Fig. 2, inset).

We incorporated PUMP and UcP into egg phosphatidylcholine liposomes and compared the H⁺/OH⁻ transport properties (Fig. 2). The fast alkalization (Fig. 2, line *a*) following valinomycin addition indicates increased H⁺/OH⁻ conductance through the incorporated proteins, as it was not observed in protein-free liposomes (Fig. 2a, line *b*). Figure 2B (line *b*) shows the inhibitory potency of externally added GTP to UcP-containing proteoliposomes. The specific activity of UcP was $7.2 \pm 1.4 \mu\text{mol H}^+$ per min per mg ($n=5$) at 28 °C, well within published data⁵. The specific activity of PUMP, determined under similar conditions, was $7.7 \pm 1.5 \mu\text{mol H}^+$ per min per mg ($n=5$). As found previously⁵, addition of 0.1 mM ATP inhibited 50% of the activity of UcP, whereas 0.05 mM GTP inhibited 80%.

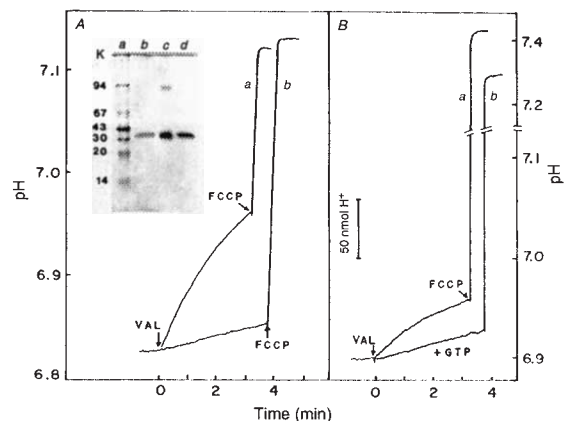


FIG. 2 Variation of external pH during H⁺ influx into vesicles prepared with or without PUMP or UcP. UcP from brown fat mitochondria and PUMP from potato mitochondria were purified as described by Klingenberg⁵ for UcP. Freshly isolated UcP or PUMP in 90 mM K-phosphate buffer pH 7.5, 2.5% decylpentaoxyethylene ether and 0.2 mM EDTA were used to dissolve ~20 mg ml⁻¹ of phosphatidylcholine (PC). Proteoliposomes were obtained after detergent removal by successive treatment with Amberlite XAD-4. External solutes were removed by filtration on a Sephadex G-25 column previously saturated with sonicated PC vesicles and eluted with 0.5 mM PIPES, 0.5 mM HEPES, 0.2 mM EDTA, 0.25 M sucrose pH 7.5. pH was monitored with a combination electrode at 28 °C with a Radiometer PHM82 pH meter equipped with a REC80Servograph. Valinomycin (VAL) (1 $\mu\text{g ml}^{-1}$) and 1.3 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were added as indicated. *A*, Vesicles of PC loaded with phosphate buffer pH 7.5, after Sephadex elution, were added to 0.5 mM HEPES, 0.5 mM PIPES, 0.2 mM EDTA, 250 mM sucrose, 5 mM choline chloride, pH 6.8. Line *a*, vesicles of PC (0.46 mM) with PUMP (3.9 mg ml⁻¹); line *b*, vesicles of PC without protein. *B*, Line *a*, vesicles of PC (0.5 mM) with UcP (2.4 mg ml⁻¹) without GTP; line *b*, with 0.05 mM GTP. Inset shows PAGE (10 μg protein) of hydroxylapatite eluate obtained from brown fat adipose tissue⁵ containing UcP (lane *c*) and potato mitochondria containing PUMP (lanes *a* and *d*). Lane *a* contains *M_r* standards.

Inhibitions of PUMP activity by 0.1 mM ATP or 0.05 mM GTP were 50% and 35%, respectively, showing that the nucleotides inhibited PUMP less than UcP. The inhibition by ATP and GTP in the reconstituted system is compatible with their observed effect in intact mitochondria (Fig. 1). Although at present we have isolated PUMP only from potato tubers, we have observed (not shown) that mitochondria of other plants show the type of respiratory control (Fig. 1) that led us to detect PUMP. As potato mitochondria do not present cyanide-resistant respiration⁶, PUMP may be important in this tuber for heat-requiring physiological events.

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The scientist as anti-hero

W. F. Bynum

The Private Science of Louis Pasteur. By Gerald L. Geison. *Princeton University Press*: Pp. 366. \$29.95, £24.95.

In the 1960s, when French schoolchildren were asked which historical figure had done the most for France, 48 per cent named Louis Pasteur. Napoleon was a distant third at 12 per cent. In no other country would such a poll have thrust a scientist into pole position. In no other country would something like *L'Année Pasteur* be commemorated as is the centenary of Pasteur's death this year in France.

But then, Pasteur is special, and not just for the country of his birth. With Newton, Darwin and Einstein, he sits as a Scientific Immortal, in even more select company than the 40 Immortals of the Académie Française, which Pasteur joined in 1882. In our own age of anti-heroes, we know that Newton was mentally unbalanced and dabbled in alchemy and prophecy; have had Einstein's dark sexual secrets made public; and have been told that Darwin lived in almost perpetual neurotic fear of a bloody working-class revolution. What of Pasteur, described long ago by Stephen Paget as "the most perfect man that ever entered the Kingdom of Science"?

Gerald Geison has been studying Pasteur for more than two decades. His 60,000-word entry on the French scientist in the *Dictionary of Scientific Biography* in 1974 remains the best general account in any language. It was based entirely on printed sources, however, and since then Pasteur's laboratory notebooks and other private papers have entered the public domain through their deposition at the Bibliothèque Nationale by Pasteur's devoted grandson, Dr Pasteur Valléry-Radot. These archival remains now permit a more intense investigation of Pasteur's laboratory life and the relationship between the private man and the public figure. *The Private Science of Louis Pasteur* is an early and important contribution to that endeavour.

We have long known that the sentimentalization of Pasteur, which began even before his death, produced no more than a caricature of the man. In that version, we have the devout and simple family man, happiest when in his laboratory, increasingly hampered by ill-health and physical frailty, wanting nothing more than the disinterested pursuit of truth and the betterment of mankind. Such an icon was useful for members of the science lobby in their battle to convince the public, philanthropists and national governments that investing in science would yield rich dividends. Pasteur's family and close

associates actively helped to foster the image; so did Pasteur himself.

By going behind the scenes, Geison discovers an altogether more tough-minded and ruthless scientist at work. He analyses four crucial stages in Pasteur's research career: the Frenchman's fascination with the optical activity of various isometric crystals such as the tartrates, and his grow-

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Pasteur, from Vanity Fair (1887).

ing conviction that such activity is always associated with the asymmetrical forces produced by living things; the famous spontaneous-generation controversy with Félix Pouchet; the anthrax work, leading to the much publicized vaccination experiments at Pouilly-le-Fort in 1881; and Pasteur's crowning glory, the development of the vaccine against rabies. To each episode, Geison adds much new information gleaned from Pasteur's laboratory notebooks, correspondence and previously overlooked printed sources. In particular, he makes frequent use of a series of reminiscences that Pasteur's nephew, Adrien Loir, published in the 1930s in an obscure public-health journal. They are of particular interest because Loir was Pasteur's technical assistant during the rabies-research period. Throughout, Geison shows that there are intriguing discrepancies between Pasteur's private science and public utterances.

These differences range from Pasteur's failure to acknowledge other investigators from whose work he had benefited to withholding details of how his vaccines had been prepared, or even which of several versions he was using. Further, he was guilty of slighting the contributions of co-workers, and of exaggerating the amount and obscuring the precise results of the experimental work that underlay the public demonstrations of anthrax and rabies vaccination. He was shrewd in his financial affairs, and certainly more commercially minded than the iconic version has it; but even in Geison's account, considerably less prone to scientific profiteering than Justus von Liebig, Emil von Behring and a number of other giants of nineteenth-century science.

Take the rabies work, for instance. The vaccination of young Joseph Meister is one of the legends of medical history. In Pasteur's own account of the episode and its scientific rationale, presented in his famous paper to the Académie des Sciences three-and-a-half months later, the drama was certainly there, alongside a quiet confidence that his animal work had fully justified the clinical experiment (for that was what it surely was) on Meister. After all, Pasteur asserted, he had successfully vaccinated about 50 dogs with his new rabies vaccine.

The reality was altogether messier. The dog experiments were neither so numerous nor so clear-cut: there had been little use of controls, several unexplained deaths among his laboratory animals, and results that were difficult to understand. Pasteur's ideas about the best way to prepare the vaccine, or even whether rabies vaccination should start with the injection of a highly virulent strain and proceed to the less virulent, or vice versa, were anything but clear when he treated Meister. Geison offers circumstantial evidence that Pasteur and his chief collaborator, Emile Roux, quarrelled over the ethics of venturing into the unknown with Meister, and less circumstantial evidence that the method of preparing rabies vaccine using dried rabbit spinal cord came from Roux. He conclusively demonstrates that Meister was not the first human to receive some form of rabies vaccine: an older man who survived, and a young girl who died, had each been given injections of earlier versions of rabies vaccines a couple of months before Meister was bitten. Pasteur never mentioned these episodes in his public statements.

All of this results in a more richly textured account of the rabies period. Similar kinds of detail are newly revealed in the case studies of earlier phases of Pasteur's research career. Whether "deceitful" (a word Geison uses often) is the best way of describing Pasteur's relationship with his colleagues and the general public is a matter of debate. In Geison's hands,

Mary Evans

Pasteur is shown to have been a consummate publicist and showman (one reason, Geison suggests, why the Americans took to him so warmly). He was also single minded, secretive and rather selfish. *Menschliches, Alzumenschliches*, as Nietzsche had it.

None of this detracts from Pasteur's historical importance, nor even, I think, from his stature as a scientist. He belongs in the Pantheon. At the same time, he would have been a great companion at the races, so good were his hunches. He gambled over tactics in scientific debate, on promising research lines to follow, on when to go public and on how much to tell the punters. He always seemed to win, even if sometimes for the wrong reasons. He is, in short, the perfect anti-hero for our anti-heroic age. □

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Planetary planting

Christopher P. McKay

The Snows of Olympus: A Garden on Mars. By Arthur C. Clarke. Gollancz: 1995. Pp. 120. £18.

TERRAFORMING — a wonderfully self-defining word that means altering another planet to make it Earth-like — is not what it used to be. Once the sole province of science fiction, terraforming — at least as applied to Mars — is now seriously discussed by space scientists. In our Solar

System, Mars is the only likely candidate. It is clear that Mars once had a different environment from the cold dry world we see today; liquid water flowed on the surface — and if once before, why not once again? And, somewhat ironically, terraforming applied to Earth is being considered as a remedy to various global ills inflicted on our planet by human activity (such as the enhanced greenhouse effect and the ozone hole). With such impetuses, technical articles on making Mars habitable have even made it into the pages of *Nature*.

In *The Snows of Olympus*, Arthur C. Clarke blends together the old fantasies of terraforming Mars with the new studies of how a planetary-scale ecosystem might develop on that planet. He is certainly the man for the job. Clarke strides through the book with one foot in the realms of the imagination and the other firmly rooted in technical reality. He briefly reviews the history of our fascination with Mars and the celebrated canals of Percival Lowell. This is followed by a personal history of how Mars was revealed in the Mariner and Viking missions. Clarke was closely involved in these projects and recalls how the first three Mariner spacecraft to reach Mars were unlucky enough to see only the part of Mars heavily cratered and Moon-like. So the river channels and mighty volcanoes were even more surprising when discovered by Mariner 9. Viking set out to search for extant microbial life and returned with only a request for further study. Clarke follows the plans for human exploration in the near future mainly by focusing on the scheme developed at the International Space Univer-

sity, which entails a crew of eight on Mars for 40 days, carried afar by nuclear rockets. Other suggested mission designs can be found in the reading list at the end of the book.

The main entrée in this book is the future Mars as it would appear after terraforming has begun. Clarke gives the correct basic outline of how terraforming would proceed but has wisely left the details to more technical publications. The first phase simply involves warming up the planet and providing a thick atmosphere of carbon dioxide. Water could flow and plants could grow, albeit slowly at first. Humans could dispense with bulky pressurized suits but would still require a source of oxygen to breathe.

Clarke effuses about the image-processing software Vistapro that allowed him to construct on his home computer images of what Mars would look like at various stages of the terraforming process. He regales us with scenes of Olympus Mons as the sands of Mars gradually yield to a green invasion. The first plants are the hardy lichens on the plains at the foot of the mountain. Then more varieties of lichens and primitive plants form a clear green ring around Olympus. The mean annual temperatures are still below freezing and there is growth only in the summer — not unlike the Antarctic. Two hundred years later, even the caldera is home to red and green lichens. In the final stages of terraforming there is a seasonally ice-free lake on the summit of Olympus and the flora consists of pines and oaks. The year is AD 2600.

The final images show a green mountain with a blue dot at the centre. Mars has become life's second home. In a sense, Mars has recaptured its past; we have restored it to the habitable state it once enjoyed. The life-forms are more advanced than those that probably inhabited Mars during its short-lived youth. Cousin Earth has shared its genome with Mars and the new life-forms on the red planet are the result of billion of years of evolution. The march of life goes on and we humans play, for once, a constructive role. *The Snows of Olympus* captures the essence of this call to adventure.

Even the sharp-eyed will find little fault in this book (although the Jet Propulsion Laboratory moves to Houston on page 18 — appropriately so given the current rush to 'reinvent' NASA — but has returned to Pasadena by page 19). It is well illustrated with both computer graphics and artistic impressions, and includes several outstanding pictures by Michael Carroll, whose representation of a terraformed Mars appeared on the cover of *Nature* 352, 489 (1991). Neither a technical work nor a work of fiction, this is a book that can be enjoyed by anybody intrigued by the idea of terraform-



Digital landscapes — a Vistapro image of a terraformed Mars in AD 2700. With the rapid warming of the planet, deciduous trees such as oaks are beginning to replace conifers such as pines on the floor of the Olympus Mons caldera.

From *The Snows of Olympus*

ing Mars and who wants to know more.

Clarke closes with an elegant assertion of the importance of space as a frontier of discovery. These are the most powerful words in this visionary book and should be read by all would-be Martian pioneers. To explore is human, and Mars is the world that beckons. □

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Breaking no new ground

William A. Shear

Invasions of the Land: The Transitions of Organisms from Aquatic to Terrestrial Life. By Malcolm S. Gordon and Everett C. Olson. Columbia University Press: 1995. Pp. 312. \$65, £49.

THE standard review volumes on the origins and adaptations of land animals are Colin Little's *The Colonisation of Land and Terrestrial Invasion*, published by Cambridge University Press in 1983 and 1990 respectively. Although otherwise rich in detail and hypotheses, these volumes contain only a sketchy treatment of the fossil record, and, of course, nothing on land plants.

It was with some anticipation, then, that I opened *Invasions of the Land*, which includes chapters by the late Everett C. Olson, an icon of North American vertebrate palaeontology. Serious disappointment awaited me. The chapters on the fossil record are so riddled with errors that I find it difficult to believe they could be Olson's final versions. For example, on page 132, late Silurian sediments are said to yield "several genera of centipedes", but on page 143 it is claimed that centipedes first appeared in the fossil record in the middle Devonian, and further down the same page they are attributed to the early Devonian. Right the first time — a single genus (undescribed) of centipede appears in late Silurian sediments. Later, a drawing of *Latzelia*, a Carboniferous centipede, is labelled as representing a "centipede arachnid", making it a member of two subphyla simultaneously. When this sort of confusion repeatedly arises, one's confidence in the rest of the book is shaken.

Heterodox opinions are expressed but not supported. Surely no authority on terrestrial ecosystems would agree that "oniscoid isopods are the most successful of terrestrial invertebrates today" as long as spiders and insects are about. The



ROUGH with the smooth — alligator snapping turtle *Macrochelys temminckii* (top) and smooth softshell turtle *Trionyx muticus muticus*. Taken from the comprehensive and detailed *Turtles of the United States and Canada* by C. H. Ernst, J. E. Lovich and R. W. Barbour. Smithsonian Institution Press, \$60.

assertion that no amphipods are adapted to strictly terrestrial life would come as a surprise to the abundant litter-dwelling groundhoppers well known from the mountains of Papua New Guinea and Japan; they are as fully terrestrial as the isopods that occur with them.

The chapter on plants by David Chapman, an algologist who for some reason is not listed as a coauthor even though he also contributes to the introductory and summary chapters, is a competent short review of plant terrestrialization. But it dwells for too long on the evidence for the close relationship between embryophytes and green algae, a subject already covered exhaustively by Linda Graham in *Origin of Land Plants* (Wiley, 1993). That book is neither cited in the text nor listed in the bibliography. In this day of rapid publication, there is little excuse for excluding an important reference published two years earlier.

Other relevant literature is also curiously handled. The description of an early Devonian archaeognath insect by C. Labandeira and colleagues (*Science* 242, 193; 1988) is referred to in the bibliography but not in the text, whereas the reverse is true for the discovery of Pridoli

terrestrial arthropods by A. Jeram and colleagues (*Science* 250, 658; 1990).

The chapters by Malcolm S. Gordon on the adaptations of extant transitional taxa are much less comprehensive than those in Little's two magisterial treatises and include few references more recent than 1990. But to me their great shortcoming is their lack of a unifying theme or viewpoint; without central organization, the material winds up as a mere list of features, taxon by taxon. There are few attempts to analyse any of the various hypotheses about terrestrialization posed by Little and others.

As to the promised "new theoretical and conceptual framework", I was unable to extract anything novel from the introductory and summary chapters. The framework seems to consist of a reaffirmation of the principle of uniformitarianism, a rejection of the relevance of cladistic methodology and a small collection of ideas that have been aired in the terrestrialization literature for at least a decade. □

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Telling it like it is

John Galloway

Dazzle 'em with Style: The Art of Oral Scientific Presentation. By Robert R. H. Anholt. W. H. Freeman: 1995. Pp. 200. £9.95 \$11.95 (pbk).

WORD has it that scientists can't communicate. The jury is still out on whether the problem is science itself. Is science just dull or is there something peculiar about scientists and their culture? Or both?

The philosopher A. J. Ayer once told me about a meeting he chaired in Oxford at which Bertrand Russell gave an address on mathematical logic. The audience, of an intellectual calibre only a place such as Oxford can muster, were stupefied by Russell's incomprehensibility. Invited by Ayer at the end of the talk to ask the great man questions, the floor remained silent. Ayer shouldered the burden and asked Russell to clarify a point that he had "failed to appreciate fully". He was rewarded by Russell's uncompromising refusal in words to the effect of "your intellectual shortcomings are hardly my responsibility". Russell's topic was difficult. But whatever his motives in giving the talk, enlightening his audience was not apparently one of them. The story is all too familiar: a complicated subject and a lecturer unwilling or unable to make it intelligible.

Faced with the same problem, Robert Anholt has not been content to sit still. He does not tell audiences how to endure scientists' talks stoically; rather, he advises science lecturers on how to communicate more effectively. If their lectures are clear, he cunningly points out, then they are more likely to persuade governments or industries to give them grants or jobs. Well, up to a point. As someone once said to me: "If your thoughts are rubbish merely, better not write too clearly".

Dazzle 'em with Style contains some good old-fashioned advice about giving lectures. It is the sort of advice that any half-decent communications consultant would give — for a hundred times the price. If the advice seems like common sense, then that's because it is: think about who you are speaking to; lead the audience carefully through the labyrinth surrounding your subject; and ensure your listeners know what question you are trying to answer and why the question is, for the moment at least, uniquely important and interesting. Then come the tricks of the trade: don't talk with your back to the audience; don't talk in a monotone; make good use of the pregnant pause; don't write on the blackboard unless you actually know how to; prepare your talk well so that it is smooth and unrushed; make sure your slides are readable and do not con-

tain too much information; and learn how to deal with questions — in particular how to savage the opposition in the nicest possible way.

As a handbook, it can't really be faulted — except in one respect. The real trouble scientists have with communication is with language itself. They often seem incapable of using it precisely or vividly. What characterizes a good talk or article is not difficult to define. Asked what makes a good poem, Robert Graves said: "It makes complete sense and says all that it has to say memorably and economically". If it demolishes the audience's preconceptions, so much the better. Mark Twain said it all in "Fenimore Cooper's Literary Offences" when he wrote that the rules of literary art require that an author shall say what he is proposing to say, not merely come near it; use the right word, not its second cousin; eschew surplusage; not omit necessary detail; avoid slovenliness of form; use good grammar; and employ a simple and straightforward style.

These points are reprinted in Herbert and Eve Clark's *The Psychology of Language* under the heading — and I joke not — "Indirect Utilisation of Utterances". As Anholt reminds us, titles matter. They provide important information. *Dazzle 'em with Style* is OK as a title, but as it says nothing about the book. The subtitle, *The Art of Oral Scientific Presentation*, is complicated, abstract and ambiguous — it could refer to what a patient does at the dentist's. What is wrong with *How to Give a Good Talk*?

This is not to say that Anholt's book is not useful. Many science lecturers would benefit from it. But the author has concentrated on what seems to me to be window dressing. Many of the best talks are given without the use of slides. Indeed, outside science, the idea of scientists needing slides is thought to be rather funny. Robert Graves also said that a good poem is written for no reasons other than poetic ones. Similarly, scientific presentations should be more than just a public relations exercise.

Sadly, very few scientists have a sense of theatre. When I was growing up in Sheffield in the late 1950s, George (now Lord) Porter, who later won the Nobel prize for his work on photochemistry, gave 'lectures' to some 2,500 local schoolchildren at a time in the City Hall. This was chemistry as pantomime: noisy, vivid, colourful — and the kids learned something, too.

Most science does not lend itself to such theatrical fireworks. But any talk can be precise, forceful and memorable. If you want to know how to speak well, listen to some good poets reading their own work or good actors reading someone else's. (Anholt gives a list of suggestions for further reading rather than a list for further listening.) And if you'd prefer to

listen to a scientist, then try the recordings of the famous series of lectures delivered by Richard Feynman at the California Institute of Technology from 1961 to 1963 (*Six Easy Pieces: Essentials of Physics Explained by its Most Brilliant Teacher* by Richard Feynman, Addison-Wesley, 1994; available on audio cassette or compact disc). There are no slides, no gestures; you don't know what he is wearing; and the sound quality is terrible. But they are superb examples of "the art of oral scientific presentation". □

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■ A revised edition of *Selling Science: How the Press Covers Science and Technology* by Dorothy Nelkin has just been published by W. H. Freeman at £11.95, \$15.95. In this provocative and probing book, Nelkin examines the complex relationships between scientists and journalists that affect American media coverage of science, both in print and on television. She reveals not only the constraints, biases and myth-making of journalists but also the surprising public-relations strategies of scientists, universities, corporations and governments. Topics now include the reporting of DNA fingerprinting, biotechnology disputes and cases of scientific fraud.

New Journals

This year, *Nature's* annual new journals review supplement will appear in the issue of 21 September. Publishers and learned societies are invited to submit journals for review, taking note of the following criteria:

- Journals that first appeared during or after June 1993 and issued at least four separate numbers by the end of April 1995 will be considered.
- Journals covering any aspect of science are eligible, although those dealing with clinical medicine and pure mathematics are excluded, as are publications of abstracts.
- Frequency of publication must be at least three times a year. The main language used must be English. Translation journals in English are, of course, eligible.
- Deadline for submission is the end of May.

When submitting journals for review, please send at least four different issues (the first, the most recent and any two others) of each title, together with full details of subscription rates (personal and institutional) and frequency of publication to: Peter Tallack, *Nature*, Macmillan Magazines Ltd, Porters South, Crinan Street, London N1 9SQ, UK. Tel: +44 (0)171 843 4567.

Progress and prospects in neutrino astrophysics

John N. Bahcall, Kenneth Lande, Robert E. Lanou Jr, John G. Learned, R. G. Hamish Robertson & Lincoln Wolfenstein

Four separate experiments to detect neutrinos from the Sun have now confirmed a deficit in the flux relative to the predictions of standard theories of nuclear physics. Future experiments with new neutrino detectors promise to reveal the explanation for this shortfall. The planned detectors may also engender a new field of astronomy, based on the observation of neutrino emission from distant, energetic astrophysical sources.

NEUTRINO astrophysics has developed from theoretical calculations to an experimental discipline over the past three decades. Solar neutrinos have been observed in four underground experiments, and a burst of neutrinos was detected from a supernova in the Large Magellanic Cloud. The observed solar-neutrino fluxes all agree with the theoretical expectations to within factors of two or three; but persistent deficits of electron-type neutrinos exist in all four solar-neutrino experiments, which has led to suggestions that neutrinos may have small masses and may oscillate between different types. New neutrino experiments are required to resolve this long-standing 'solar-neutrino problem'.

In the 1920s and 1930s, scientists developed the theoretical basis for understanding how the Sun shines. They proposed^{1,2} that nuclear fusion reactions among light elements occur near the centre of the Sun and provide the energy that the Sun has emitted for four-and-a-half billion years. While most of this energy ends up as electromagnetic radiation from the surface, approximately three per cent is believed to be emitted directly from the centre of the Sun in the form of neutrinos³. In a pioneering experiment that has been going on for 25 years⁴, Davis and collaborators first detected solar neutrinos using radiochemical techniques and a cleaning fluid (perchloroethylene) as a target.

Over the past several years, there has been striking confirmation of the astronomical predictions of solar neutrinos. The Japanese water-based Čerenkov detector Kamiokande has identified solar neutrinos by their directionality, measured by observing the direction of recoil electrons from neutrino interactions⁵. Recently, two radiochemical experiments using gallium (the Gallex experiment^{6,7} in Italy and the Sage experiment^{8,9} in Russia) have detected the copious low-energy (below 400 keV) neutrinos that are the primary component of the solar-neutrino flux.

In February 1987, neutrinos from supernova 1987A were detected as a series of pulses in two water Čerenkov detectors (Kamiokande¹⁰ in Japan and IMB¹¹ in the United States). The neutrinos arrived, as expected, a few hours before the light from the explosion. A supernova represents one of the most awesome events in astronomy. Type II supernovae are believed to result from the sudden gravitational collapse of a star that has completed cycles of nuclear burning. The energy calculated to be produced from this collapse is almost 1,000 times larger than that observed as light. Theory indicates that more than 99 per cent of the energy is emitted in the form of neutrinos¹².

The definitive detection of solar neutrinos, and the detection of neutrinos from SN1987A, are two of the most remarkable scientific discoveries of the past decade. They provide dramatic confirmation of fundamental theories concerning stellar interiors. The detection of solar neutrinos demonstrates that fusion energy is the basic source of energy received from the Sun. The observations of solar and supernova neutrinos open up a new area of science: neutrino astrophysics.

The observation of astrophysical neutrinos makes it possible to probe the innermost regions of stars, dense regions from which light cannot escape. Because of their very small interaction probabilities, neutrinos can escape from these inner regions; for the same reason, of course, it is difficult to detect these elusive particles.

The motivation for proposing^{13,14} the first practical solar-neutrino experiment was astrophysical, to test directly the hypothesis that stars shine and evolve because of nuclear fusion reactions in their interiors. However, it has become apparent in recent years that solar neutrinos provide a beam of elementary particles that can be used to investigate fundamental physics, in particular to study intrinsic neutrino properties.

Of particular importance is the question of whether neutrinos have mass and whether they transform (oscillate) from one type to another¹⁵⁻¹⁸. (There are believed to be three types of neutrinos: electron-type (ν_e), muon-type (ν_μ) and τ -type (ν_τ).) Only electron neutrinos are created in the Sun, but all types are believed to be emitted from supernovae. The question of neutrino mass is of fundamental importance for particle physics and for cosmology. In the case of particle physics, neutrino mass may provide a clue to the problem of the origin of the masses of all particles. So-called grand unified theories¹⁹ of particle physics suggest that all interactions (strong, electromagnetic and weak) are unified at a large energy scale and that the neutrino masses are inversely proportional to this scale. Two related ways to probe this very high energy scale are the search for proton decay and the search for very small neutrino masses.

Our basic ideas about cosmology that explain the microwave background radiation predict a similar background of relic neutrinos. If the heaviest of these neutrinos, usually taken to be ν_τ , has a mass above a few electron volts, relic neutrinos could be an important component of dark matter. In this case, ν_μ with a smaller but still non-zero mass, could provide an explanation for the solar neutrino problem.

The only available way to study very small neutrino masses is through the possibility of oscillations that transform one type of neutrino into another. For neutrino masses greater than 10^{-2} eV such oscillations have been looked for in experiments (not discussed here) using neutrinos produced in the Earth's atmosphere by cosmic rays, or those produced in the laboratory by reactors or accelerators. For smaller neutrino masses (ν_μ or ν_τ with mass in the range 10^{-2} to 10^{-5} eV) the only available possibility is to use solar neutrinos^{15,16}, with oscillations depleting the flux of ν_e by converting some of them into ν_μ or ν_τ . Of particular interest is the possibility that the oscillations may be enhanced^{17,18} by the coherent interaction of the neutrinos with the solar material. This coherent interaction is called the MSW (Mikheyev, Smirnov and Wolfenstein) effect.

The observations made with the four experiments performed so far indicate fluxes of electron neutrinos that are consistently

less than the results of astrophysical calculations. In fact, comparisons between the rates of different experiments suggest (independent of astrophysical uncertainties) that some previously unaccounted-for physical process, perhaps involving non-zero neutrino mass, may be occurring. The experiments carried out so far are the first pioneering experiments and final conclusions require additional experiments that are currently being developed.

We will also consider here some of the future experiments designed to observe neutrinos of much higher energies, six to nine orders of magnitude larger than those of solar neutrinos. The potential sources of higher-energy neutrinos, the techniques for their detection, and the scientific justifications for the research are all very different from the solar case. Unlike the Sun, possibly astrophysical sources of high-energy neutrinos are remote and not well understood; estimates of their neutrino fluxes are necessarily speculative. Detectors of high-energy neutrinos are complementary to detectors of solar neutrinos, being optimized for large areas rather than for low thresholds.

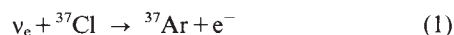
Candidates for sources of high-energy neutrinos include such exotic but relatively nearby objects as neutron stars and black holes in the Galaxy, as well as the nuclei of distant but highly luminous galaxies. Because we observe cosmic rays too energetic to be confined within the Galaxy²⁰, we know that there exist enormously energetic processes that might well be sources of high-energy neutrinos. Will any of these sources produce detectable numbers of high-energy neutrinos. We will never know until we look.

Continuing solar-neutrino experiments

There is no single, best type of detector that can be used to observe solar neutrinos. The energies of the neutrinos vary over a range of one-and-a-half orders of magnitude and the fluxes from the most important neutrino sources vary by four orders of magnitude. According to standard solar-model calculations, the numerous neutrinos from the initiating proton-proton reaction that have energies less than 0.4 MeV have a flux of about $6 \times 10^{10} \text{ cm}^{-2} \text{ s}^{-1}$; the rare, higher-energy neutrinos from ^8B -decay with energies up to 14 MeV have a flux of about $6 \times 10^6 \text{ cm}^{-2} \text{ s}^{-1}$. Recently published solar models, eleven in number and each constructed using a different computer code and different input physics, give²¹ fluxes for those neutrinos less energetic than 1 MeV which are the same to better than 10%.

Figure 1 shows calculated solar neutrino spectra from different neutrino sources. Because of the wide range in energies and in fluxes, a set of detectors with sufficient complementarity and some redundancy is required to untangle the different aspects of neutrino physics and of solar physics.

The chlorine experiment. In this remarkable experiment⁴, neutrinos are detected using a large tank containing 615 tons of perchloroethylene, C_2Cl_4 . The target isotope, ^{37}Cl , can capture a neutrino, producing a radioactive isotope ^{37}Ar by the following process:



This reaction can occur for electron neutrinos with energy greater than 0.8 MeV. The neutrino reactions are registered by counting the radioactive nuclei, ^{37}Ar . To avoid extraneous (background) interactions caused by cosmic rays incident on the surface of the Earth, the experiment is conducted 1,500 metres underground in the Homestake gold mine in Lead, South Dakota. After about two months in the solar-neutrino 'sunshine', the build-up of ^{37}Ar atoms from neutrino capture by ^{37}Cl is approximately balanced by the loss due to decay. At this point, the standard solar model²²⁻²⁴ predicts that only about 54 atoms of ^{37}Ar are present in the 615 tons of C_2Cl_4 . Over the past two decades, more than 100 extractions of ^{37}Ar have been performed. After correction for detection efficiency, the average number of ^{37}Ar atoms in the detector at extraction is observed to be only 17, corresponding to a solar-neutrino-induced production rate

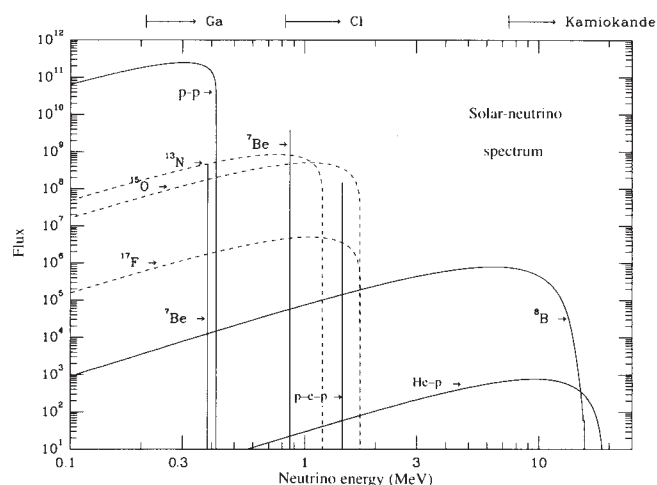


FIG. 1 Energy spectrum of neutrinos predicted by the standard solar model³. The neutrino fluxes from continuum sources (such as p-p and ^8B ; see text) are given in units of number per cm^2 per second per MeV at 1 AU; the line fluxes (p-e-p and ^7Be) are given in number per cm^2 per second. Solid lines, spectra from the p-p process; dotted lines, spectra from the CNO process. The arrows at the top of the figure indicate the energy thresholds for the current neutrino experiments. (p-e-p neutrinos are formed when two protons and an electron combine to form a deuterium nucleus and a line neutrino; He-p neutrinos are formed by the reaction $^3\text{He} + p \rightarrow ^4\text{He} + e^+ + \nu_e$).

of 0.5 atoms per day, far fewer than the 1.5 atoms per day expected on the basis of the standard model. In terms of the solar neutrino unit, SNU (pronounced 'snew', $1 \text{ SNU} = 10^{-36}$ interactions per target atom per second), the observations yield $2.55 \pm 0.25 \text{ SNU}$, about one-third of the prediction of the standard model. For two decades, this discrepancy between the measured chlorine event rate and the event rate predicted by the standard model constituted the well-known 'solar neutrino problem'.

The Kamiokande experiment. Like a supersonic aircraft creating a sonic boom, a charged particle moving through matter faster than the speed of light in that matter gives off a conical shock wave of light called Čerenkov radiation. Sensitive photomultiplier tubes can record the flashes of light that betray the presence and direction of these high-speed particles. With the aid of special large photomultiplier tubes, experimenters working in a mine in the Japanese alps and using a 3,000-ton detector of ultra-pure water (680 ton fiducial volume), called Kamiokande, were able to detect electrons that had been struck by neutrinos from the Sun⁵. Figure 2 shows the inside of the Kamiokande detector. The reaction that was observed is



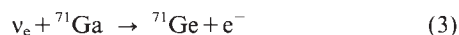
At the energies of interest for solar-neutrino detection, reaction (2) is about six times more likely for ν_e than for ν_μ or ν_τ . Despite a significant background from radioactivity of the rocks in the mine, from radioactivity in the water, and from cosmic-ray-induced events, the neutrinos were unambiguously detected because the electrons struck by neutrinos recoiled preferentially in the direction from the Sun to the Earth. Kamiokande, the first 'active' (electronic rather than radiochemical) solar-neutrino experiment, showed two very important things: first, the detected neutrinos do indeed come from the Sun, and second, the observed flux above an electron-detection threshold of about 8 MeV is about half as large as predicted by the standard solar model. The discrepancy between prediction and observation is less severe at energies above 8 MeV (the Kamiokande experiment) than it is for all energies above 0.8 MeV (the chlorine experiment).

The predicted counting rate for any particular solar-neutrino experiment depends on calculations that are usually based upon

a standard solar model. Solar models are constrained by measurements of the solar luminosity, age, chemical composition and thousands of seismological frequencies. Nevertheless, the predicted neutrino emission rates cannot be tested independently of solar-neutrino experiments. Changes in the input data for the standard solar model could in principle be made just so as to fit the results of any one of the existing experiments. However, it appears essentially impossible to explain simultaneously the results of the chlorine and the Kamiokande experiments by adjusting input solar data^{21–27}. Just the ^8B neutrinos that are observed in the Kamiokande experiment correspond to an expected counting rate in the chlorine experiment of (3.21 ± 0.46) SNU compared with the observed rate of (2.55 ± 0.25) SNU. But in the chlorine experiment, the solar model predicts an additional 1.2 SNU from the much more reliably calculated lower-energy neutrinos (^7Be and $p\text{-}e\text{-}p$ neutrinos, see Fig. 1). This suggests that the lower-energy neutrinos might be preferentially depleted by some new weak interaction process (which could be the MSW effect discussed below).

The gallium experiments. The neutrinos produced by the most basic fusion reaction in the Sun (the fusion of two protons, the so-called $p\text{-}p$ reaction) are too low in energy to be detected by either the chlorine or the Kamiokande experiment. As the $p\text{-}p$ neutrinos are predicted to be the most numerous neutrinos emitted by the Sun, and as their flux is more reliably predicted than any other neutrino flux³, the measurement of the terrestrial flux of $p\text{-}p$ neutrinos has long been seen as an essential goal of solar-neutrino astronomy. The possibility of using gallium as a detector for these neutrinos was suggested almost 30 years ago²⁸ and a design for the experiment was developed²⁹. Only in the past six years has it proved possible to carry out this important experiment.

The detector reaction is



which has an energy threshold of 0.2 MeV. The gallium experiments operate similarly to the chlorine experiment, extracting and counting radioactive atoms of ${}^{71}\text{Ge}$. The standard-model prediction is that after a month of operation about 16 atoms of ${}^{71}\text{Ge}$ will be present in 30 tons of gallium.

One of the gallium experiments, Gallex^{6,7}, is a European–American–Israeli collaboration operating in the Gran Sasso tunnel near Rome. Gallex uses 30 tons of gallium in a $\text{GaCl}_3\text{--HCl}$ solution. The other experiment, Sage^{8,9} (the Soviet–American gallium experiment) is operating in a subterranean laboratory under Mount Andyrchi in the Caucasus Mountains, in the southern region of Russia. Sage currently uses 60 tons of molten gallium metal.

The fact that the detectors use gallium in two very different chemical environments—the $\text{GaCl}_3\text{--HCl}$ solution (Gallex) and the metallic form (Sage)—provides a consistency check on the results. The current Gallex results are (79 ± 12) SNU and the current Sage results are (74 ± 14) SNU. These results also are significantly below the standard model prediction, which is (132 ± 7) SNU for gallium detectors. The Gallex detector response to neutrinos has been experimentally checked³⁰ using a source (${}^{51}\text{Cr}$) of 1.7 MCi that emits neutrinos of 750 keV and 430 keV from electron capture. A similar check is planned for the Sage detector.

Summary. As time has passed and more experiments have been performed, the solar-neutrino problem has not gone away. Indeed it appears that, independent of detailed solar-model calculations, there is no combination of the expected neutrino fluxes that fits all the available data.

On the other hand, the results of all four experiments (chlorine, Kamiokande, Gallex and Sage) can be explained^{31,32} if either ν_μ or ν_τ has a mass of about 0.003 eV and has a small amount of mixing with a lighter ν_e . In this case, the ν_e produced at the centre of the Sun are transformed (with a transformation probability that depends upon the energy of ν_e) into ν_μ or ν_τ as

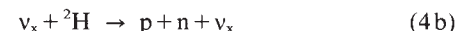
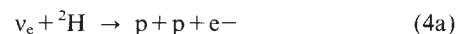
they pass through the material medium of the Sun (the MSW effect^{17,18}). Only future experiments can determine if this is indeed the correct explanation.

Solar-neutrino experiments under construction

Solar-neutrino detectors fall into two classes, ‘active’ (like Kamiokande) and ‘radiochemical’ (like chlorine and gallium). Active detectors provide information about the arrival time, direction and energy of individual neutrinos, and in some cases the neutrino type (for example, electron-type or muon-type), but are complex and are often faced with persistent backgrounds. Radiochemical detectors are sensitive only to electron-type neutrinos (that is, they specify uniquely the neutrino type) and provide a single number for the rate of detection of all neutrinos above an energy threshold, a number that is averaged over a period of time comparable to the mean life (typically weeks or months) of the radioisotope that is produced.

Here we describe the three active and one radiochemical solar-neutrino detectors that are under construction. All of these detectors will have much higher event rates; several thousand events per year for the active detectors. The expected high event rates will make it possible to search for time dependence in the neutrino flux, in particular the expected seasonal variation due to the orbital eccentricity of the earth (an effect of $\sim 7\%$, peak-to-peak).

The Sudbury neutrino observatory (SNO). Deuterium (‘heavy hydrogen, ${}^2\text{H}$, with a neutron and a proton in its nucleus) is an excellent target for neutrinos. Two neutrino interactions can occur³³:



Reaction (4a) can only be induced by electron-type neutrinos, whereas the cross-section for reaction (4b) is the same for all three types. If more neutrinos are detected via reaction (4b) than by reaction (4a), that would be direct evidence that some electron-type neutrinos have oscillated into neutrinos of some other type.

The SNO is located near Sudbury, Ontario in one of the deepest mines in the western hemisphere (a nickel mine belonging to the INCO company). A large cavern has been excavated 2,070 m underground to hold a detector consisting of 1,000 tonnes of heavy water. An artist’s version of the detector is shown in Fig. 3. When SNO begins operation in 1996, solar neutrinos are expected to be detected at the rate of more than 10 counts per day via reactions (4a) and (4b). In addition to the unique reactions of neutrinos with deuterium, SNO will observe the same neutrino–electron scattering process that is detected by Kamiokande.

If electron-type neutrinos oscillate into one of the other known neutrino types as they travel from the interior of the Sun to the terrestrial detector, this will be revealed by the comparison of the rates in the two deuterium reactions, and, if the oscillation parameters are favourable, also by its distinctive effect on the shape of the observed electron energy spectrum in reaction (4a). These potential signatures of new physics are independent of solar models and solar physics.

Superkamiokande. The Superkamiokande experiment³⁴, a much-improved version of the current Kamiokande experiment, utilizes 50,000 tonnes of ultra-pure ordinary water. This immense detector will, beginning in 1996, provide a 30-fold increase in the observed rate of neutrino–electron scattering events. The change in the shape of the neutrino energy spectrum predicted by the MSW effect may be observable in this experiment via the spectrum of recoil energies of the scattered electrons. Both Superkamiokande and SNO detect only those neutrinos with energies above 5 MeV.

Borexino. This will be the first active detector with the goal of observing the intense flux of low-energy neutrinos produced by

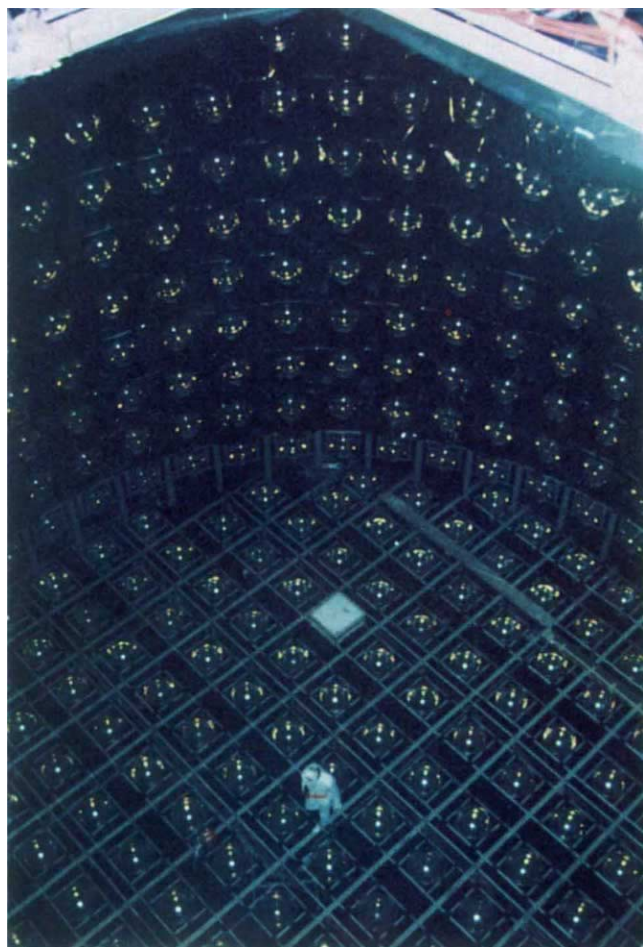


FIG. 2 The inside of the Kamiokande detector, as seen from the top. This picture was taken in 1984, when we modified the detector toward a detection of solar neutrinos. The large Čerenkov detectors that make the experiment feasible are visible on the sides and bottom of the tank, now filled with pure water (photo supplied by Y. Totsuka, Institute for Cosmic Ray Research, Tokyo University).

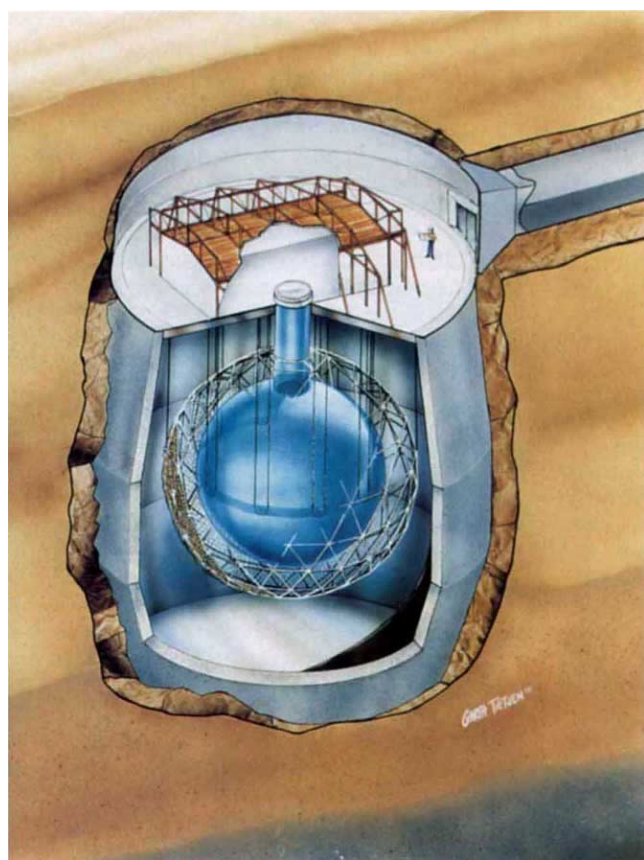


FIG. 3 Artist's impression of the Sudbury neutrino observatory, now nearing completion in Sudbury, Ontario, Canada. Located 2,100 m beneath the surface, in the INCO nickel mine, the solar-neutrino detector consists of 1,000 tonnes of heavy water (D_2O) in a thin acrylic sphere. Čerenkov light from fast charged particles knocked out by neutrinos is detected in 9,500 photomultipliers surrounding the heavy water. To screen out radiation from the rock, the cavity is filled with 7,000 tonnes of purified light water. (Photo supplied by R. Hamish Robertson, University of Washington.)

electron capture on beryllium nuclei (see the 0.86-MeV ^7Be neutrino line in Fig. 1). The Borexino detector³⁵ is to be constructed with standard liquid scintillator materials, except that the requirement for radio purity (uranium and thorium less than $\sim 10^{-16} \text{ g g}^{-1}$) is unprecedentedly stringent. If the MSW interpretation is correct, then the observed flux of ^7Be neutrinos will be greatly reduced relative to the predictions of the standard solar model.

Iodine. An iodine radiochemical detector (all ^{127}I) will be somewhat similar to the existing chlorine detector but, per target atom, is expected to have a higher counting rate³⁶. A modular 100-ton iodine detector is now under construction in the Homestake mine near the present chlorine detector and will begin operating in mid-1995.

Calibration experiments will be performed to measure the probability for neutrino-induced conversion of ^{127}I to ^{127}Xe . These experiments are necessary to determine the relative sensitivity of the detector to ^7Be and ^8Be neutrinos and to interpret the total rate. Expansion of the present iodine detector may be proposed after operating experience is obtained with the current detector, and after the neutrino cross-sections are measured.

Developing new solar neutrino detectors. Additional solar-neutrino detectors are required to obtain more complete information on the different portions of the solar-neutrino spectrum.

The 5,000 ton Icarus detector³⁷ will be installed in the Gran Sasso laboratory and is planned to be operational in 1998. Like the chlorine detector, Icarus is based on an inverse β -process,

$\nu_e + {}^{40}\text{Ar} \rightarrow e^- + {}^{40}\text{K}^*$, but operates in real time and is sensitive only to ^8B neutrinos. The detector uses liquid argon with the goal of measuring the recoil electrons and the decay γ -rays (from the ${}^{40}\text{K}$ excited state, ${}^{40}\text{K}^*$) that are produced by neutrino capture.

A major future goal is to measure the energy and arrival time of the lowest energy neutrinos, the p-p neutrinos which are detected by the radiochemical method in the gallium experiments. The proposed Heron detector³⁸ is based on the ballistic propagation of rotons (sound excitations) in liquid helium in the superfluid state with detection of the heat pulses by an array of bolometers. The goal is to achieve a threshold of the order of 10 keV, suitable for measuring the recoil electron spectrum and rate of neutrino-electron scattering from both p-p and ^7Be neutrinos. The proposed Hellaz detector³⁹ also would detect neutrino-electron scattering in helium, but in high-pressure low-temperature gas.

High-energy neutrinos

The higher-energy neutrinos that are most accessible experimentally have energies above 1 TeV ($=10^{12} \text{ eV} = 10^6 \text{ MeV}$). Astrophysical neutrinos with these energies can be produced by high-energy collisions among nuclei or between protons and photons that produce secondary particles (pions and muons), which have neutrinos among their decay products. Such collisions are believed to take place when matter falls into a neutron star from a companion star in a double-star system, and are also expected

on a large scale at the centres of active galactic nuclei (AGN). However the flux of high-energy neutrinos^{40,41} from such sources cannot be calculated with accuracy. It is only by searching for these neutrinos that we can probe the processes taking place deep in the interior of these (and other) astrophysical systems.

High-energy neutrinos can be detected through the reaction of a muon neutrino with a nuclear particle, leading to the neutrino being transformed into a charged muon. A charged muon with an energy of several TeV will travel many kilometres in earth or in water. The neutrino target volume is thus the area of the muon detector times the range of the muon. Target volumes of billions of tons may be achieved this way. The fast-moving muons can be detected by observing the blue flash of Čerenkov light (see above discussion of the Kamiokande experiment) that they produce when traversing a transparent medium, specifically water or ice. Detectors with effective areas of a few hundred square metres have been built in mines, four new instruments are being built with areas in the 20,000-m² range, and plans are being discussed for one or more instruments in the 1-km² class⁴².

The detectors that have produced data so far, like the Kamiokande detector and the Macro detector in the Gran Sasso, have observed neutrinos with energies of 10⁵–10⁷ eV produced by cosmic rays in the atmosphere. However, no neutrinos from sources outside the Solar System have been observed with the exception of the observations of SN1987A. Estimates⁴⁰ of the potential fluxes of high-energy neutrinos from sources such as AGN suggest that a surface area greater than 0.1 km² is necessary for meaningful observations. Furthermore, as the expected signal-to-noise ratio improves greatly with the increasing threshold up to TeV energies, detectors of great thickness are desirable. It is generally agreed these requirements can only be met by the use of large bodies of water such as lakes, the ocean or polar ice.

The first instruments designed specifically for high-energy neutrino detection are now under construction in Siberia in Lake Baikal⁴³ off Hawaii in the deep ocean (Dumand⁴⁴), at the South Pole in the deep polar ice (Amanda⁴⁵), and in the Mediterranean near Pylos, Greece (Nestor⁴⁶). All these instruments aim at detection areas of the order of 20,000 m² and an angular resolution of the order of 1° for TeV muons.

It is expected that high-energy neutrino astronomy will require a telescope with an effective muon-detecting area of at least 1×1 km. Two of the principal goals for a kilometre-scale neutrino telescope are: (1) the search for neutrinos from the annihilation in the Sun of weakly interacting dark-matter particles (with masses in the GeV–TeV range), and (2) the observation of individual AGN with good statistics. Studies of neutrino oscillations may also be possible⁴⁰. The four instruments presently under construction represent an increase of about 50 times in area from previous mine-based detectors; another increase of 50 times is needed to reach the desired scale.

What next?

Solar-neutrino experiments carried out over the past thirty years have closed an important chapter in the history of science that began in the early part of this century. We now have conclusive, direct evidence for nuclear-fusion reactions that occur among light elements in the centre of the Sun. At the same time, the Sun provides a unique source of neutrinos for studying the possibility of very small neutrino masses.

Solar-neutrino experiments therefore tell us about both elementary-particle physics and the details of nuclear fusion within the innermost region of the nearest star.

Experiments that are currently underway have the potential to establish—independent of theoretical calculations carried out with solar models—whether new physics is required to explain the solar-neutrino observations. If the number of neutrinos that are observed by the SNO in reaction (4b), which can be induced by any of the known types of neutrinos, exceeds the number

observed in reaction (4a), which can only be initiated by electron-type neutrinos, this will be direct evidence for neutrino oscillations. The measurements of a neutrino energy spectrum by the SNO and the Superkamiokande experiments will test whether the spectrum of the higher-energy (⁸B) solar neutrinos that arrive at Earth from the Sun is the same as the energy spectrum of neutrinos from the same radioactive isotope observed in the laboratory or has been distorted by neutrino oscillations. But it is possible that even if oscillations take place, their effect on the ⁸B neutrinos, the only ones detected by SNO and Superkamiokande, may be too small to be detected. Fortunately, theoretical calculations of the MSW effect imply that the deficit of electron-type neutrinos is much larger for the lower-energy ⁷Be neutrinos; if this is correct, the flux detected in Borexino will be much smaller than predicted by the standard model.

Solar-neutrino experiments are fundamental both for physics and for astronomy. It is essential that we do not rely on just a few experiments, because the history of science has shown that systematic uncertainties can sometimes lead to mistaken conclusions unless results are checked by performing measurements in different ways. This is particularly important when the measurements are as intrinsically difficult as they appear to be for solar-neutrino experiments.

The timing of SN1987A was extremely fortunate, as the water Čerenkov detectors had only been operating for a few years. An important lesson of that supernova neutrino detection is that neutrinos could reveal supernovae in our Galaxy even if the light is obscured by the Galaxy itself. It is very desirable that a number of detectors are ready to detect neutrinos from the next supernova in our Galaxy.

The past fifty years have seen the extension of astronomy from the optical spectrum to the whole range of the electromagnetic spectrum (radio waves to γ-rays). In this process, many new aspects of astronomy have been uncovered. Neutrino astronomy can provide a unique window by means of which one can see into the deepest interiors of stars and galaxies. So far the only sources detected are the Sun and supernova 1987A. Neutrino astronomy is an exciting frontier for exploration in the twenty-first century. □

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ARTICLES

Disruption of imprinting caused by deletion of the *H19* gene region in mice

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The imprinted *H19* gene, which encodes an untranslated RNA, lies at the end of a cluster of imprinted genes in the mouse. Imprinting of the insulin-2 and insulin-like growth factor 2 genes, which lie about 100 kilobases upstream of *H19*, can be disrupted by maternal inheritance of a targeted deletion of the *H19* gene and its flanking sequence. Animals inheriting the *H19* mutation from their mothers are 27% heavier than those inheriting it from their fathers. Paternal inheritance of the disruption has no effect, which presumably reflects the normally silent state of the paternal gene. The somatic overgrowth of heterozygotes for the maternal deletion is attributed to a gain of function of insulin-like growth factor 2, rather than a loss of function of *H19*.

GENOMIC imprinting in mammals is an epigenetic process by which the two parental alleles of a gene are differentially expressed^{1,2}. Four of the eleven genes known to be subject to imprinting have been mapped to a ~350 kilobase (kb) region of DNA on the distal portion of mouse chromosome 7 (Fig. 1a). These four genes include *meth-2*, which codes for one of a family of helix-loop-helix proteins, and is expressed from the maternal chromosome in spongiotrophoblasts of the mouse placenta^{3–5}. The insulin-2 gene (*Ins-2*) is paternally expressed in the yolk sac of the mouse embryo, but biallelically expressed in the pancreas⁶. The *Igf2* gene, encoding insulin-like growth factor 2 which promotes embryonic growth in rodents, lies immediately downstream of *Ins-2* and is expressed only from the paternal allele in most tissues⁷. At the end of the cluster lies the *H19* gene, an unusual gene that codes for a non-translatable RNA^{8,9}. Its expression is exclusively maternal¹⁰.

The clustering of four imprinted genes raised the possibility that imprinting, like X-chromosome inactivation, is controlled by a signal that can act on several genes. As the temporal and tissue-specific patterns of expression of the *Igf2* and *H19* genes are essentially identical^{11,12}, it seemed likely that their imprinting at least could be linked. We have proposed a mechanism that relies on competition between their promoters for a set of shared regulatory elements such as enhancers^{13,14}. We have also sug-

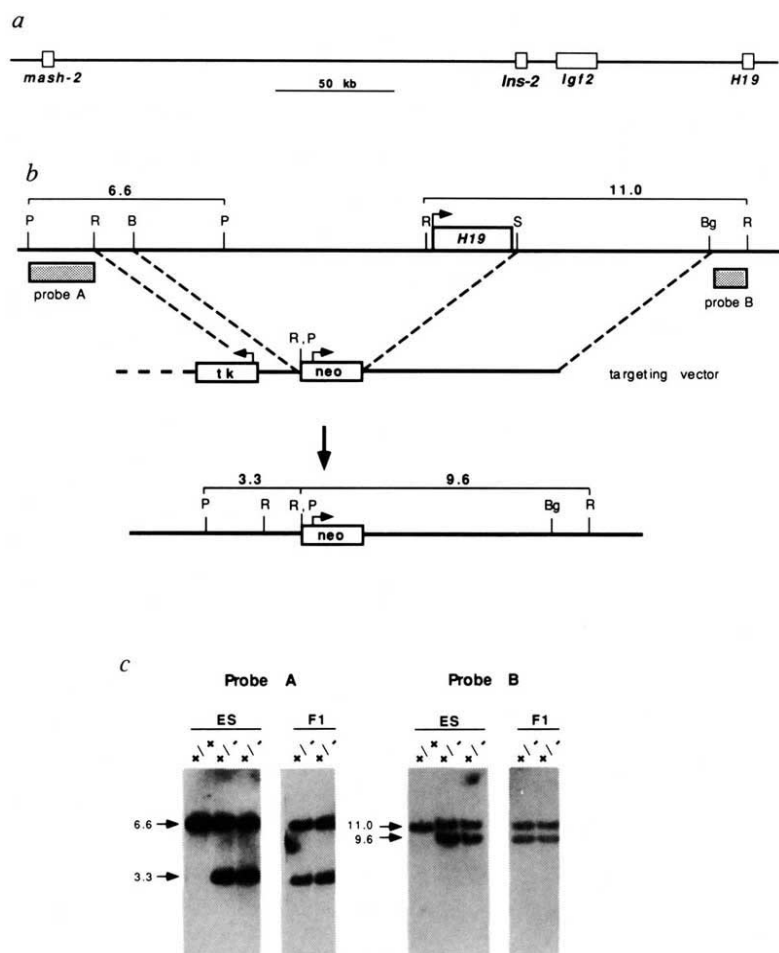
gested that paternal-specific DNA methylation around the *H19* gene promoter^{13,15}, established in the male gametes, acts as the initial imprinting mark. Its function is to block the interaction of the paternal *H19* gene with the enhancers, thereby making them available to engage the paternal *Igf2* gene. The evidence that paternal-specific DNA methylation plays a central role in the imprinting of both *H19* and *Igf2* comes from the observation that the paternal *H19* gene is activated and the paternal *Igf2* gene is silenced in an embryo deficient in DNA methyltransferase¹⁶. Furthermore a subset of the paternal-specific DNA methylation at both *H19* and *Igf2* that is inherited from sperm is retained throughout early embryogenesis, a requirement for the mark^{17,18}. On the maternal chromosome, where both genes are under-methylated and there is no sign of an epigenetic mark, we considered that *H19* could be preferentially transcribed, either because it has an inherently stronger promoter or because it is closer to the regulatory elements. This model explains how a single signal can mediate the reciprocal imprinting of the two genes.

Targeted deletion of the *H19* gene

To test the dependence of *Igf2* imprinting on the *H19* gene, we used a gene-targeting approach to generate a strain of mice carrying a deletion mutation spanning the structural *H19* gene itself (3 kb) and 10 kb of 5' flanking sequence (Fig. 1 legend).

FIG. 1 Deletion of the *H19* gene in the mouse. *a*, The positions of the imprinted genes *mash-2*, *Ins-2*, *Igf2* and *H19* on mouse chromosome 7 (refs 4, 42). *b*, Strategy for targeting the *H19* gene in ES cells. A targeting vector (middle) containing, from left to right, Bluescript KSII vector (dashed line), the herpes simplex virus thymidine kinase (*tk*) gene, a 1.1-kb *EcoRI*–*Bam*HI fragment from –11 to –9.9 kb of the 5' flank of the *H19* gene, the PGKneoBpA gene⁴³, and a 6.5-kb *Sall*–*Bgl*II fragment from +3 to +9.5 kb from the 3' flank of the *H19* gene, was introduced into ES cells. The correctly targeted integrants were identified by Southern blot analysis using probes A and B (boxes) which detect different-sized bands in the untargeted (top) and targeted (bottom) loci. The restriction endonuclease recognition sites are labelled as follows: P, *Pst*I; R, *Eco*RI; B, *Bam*HI; S, *Sal*I; Bg, *Bgl*II. Arrows indicate the direction of transcription. *c*, Identification of correctly targeted clones. Genomic DNAs from individual colonies of untargeted (+/+) and targeted (+/–) ES cell lines and F₁ animals derived from breeding of male chimaeric mice to C57BL/6J females were digested with *Pst*I and hybridized to radiolabelled probe A, a 2.2-kb *Pst*I–*Eco*RI fragment which detected the 6.6 kb and 3.3 kb *Pst*I fragments corresponding to the wild-type and mutant alleles, respectively. DNA was also digested with *Eco*RI and hybridized to probe B, a 1.0 kb *Bgl*II–*Eco*RI fragment from the 3' end of the gene which detected 11.0- and 9.6-kb fragments corresponding to the wild-type and mutant alleles, respectively.

METHODS. The targeting vector was linearized at a unique *Sall* site, leaving the vector sequences adjacent to the *tk* gene. CCE ES cells⁴⁴ were grown on STO feeder cells, were collected and 8 µg of targeting vector were added per 10⁷ cells. Cells were electroporated in 1X Hank's buffered saline at 960 µF and 200 V, and 24 h later, 0.2 µM 1-(2-deoxy,2-fluoroarabinofuranosyl)-5-iodouracil (FIAU) (gift from Bristol Meyers-Squibb) was applied to the cells, followed by 200 µg ml^{–1} (effective concentration) G418 12 h later. Selection with FIAU + G418 gave 17-fold fewer colonies than a control plate with G418 selection alone. Doubly resistant colonies were picked 8–9 d after electroporation and expanded for analysis. DNA was prepared, digested with either *Pst*I or *Eco*RI, separated on a 1% agarose gel, transferred to nitrocellulose filters and hybridized to probe A or B labelled by random hexamer labelling^{45,46}. Out of 400 clones screened, 2 correctly targeted clones were identified, expanded and injected into e3.5 C57BL/6J blasto-



cysts, where e0.5 is the morning after conception. One clone gave a maximum coat colour contribution of ~50%, and a germline contribution of ~1% in chimaeras. The other cell line gave a coat colour contribution of 90–100% in most chimaeras and a contribution to the germ line of 50–100%.

As the paternal-specific methylation domain of the *H19* gene extends from –5 kb to +3 kb of the gene^{13,15}, and the sequences required for *H19* imprinting have been localized to a domain between –4 kb and +11 kb surrounding the *H19* gene¹³, we reasoned that its putative imprinting signals would be disrupted by the deletion. In the mutants, the deleted DNA segment was replaced by a neomycin-resistance gene (*neo*^r) under the control of the phosphoglycerate kinase-1 (*pgk-1*) promoter and its own upstream activation domain^{19,20}.

Paternal Inheritance of *H19* deletion

As the paternal allele of *H19* is silent, paternal inheritance of the *H19* deletion should have no phenotypic consequences. To test this, heterozygous *H19* males were crossed to wild-type females of a strain that carries *Mus castaneus* alleles of *H19* and *Igf2* on a C57BL/6J background, B6(CAST-*H19*). In the progeny of this cross, the parental origin of the *Igf2* transcripts could be distinguished by an allele-specific RNase protection assay that detects allelic differences in the 3' untranslated region of the gene (J. R. Saam and S.M.T., unpublished results). When RNA from neonatal liver was assayed, the maternal *H19* allele was transcribed, as expected, whereas the paternal expression of *Igf2* was unaffected by the *H19* deletion in *cis* (Fig. 2a). Unlike

the paternal gene in wild-type animals, the *neo*^r gene was expressed when inherited paternally, indicating that the targeted locus did not harbour a residual imprinting signal that could act negatively on the marker gene.

Maternal Inheritance of *H19* deletion

To investigate the reciprocal situation, heterozygous *H19* females were crossed to males of the B6(CAST-*H19*) strain. Wild-type neonatal pups from this cross expressed only the paternal *M. castaneus* allele of *Igf2*, whereas their heterozygous littermates expressed both paternal and maternal *Igf2* alleles in all tissues examined (Fig. 2b). Using a quantitative polymerase chain reaction (PCR) assay, the levels of *Igf2* RNA in the same neonatal RNA samples were determined (Table 1). This assay revealed a tissue-specific difference in the degree to which *Igf2* RNA levels increased in the heterozygous progeny relative to wild-type levels. That is, liver and gut gave a less than 2-fold increase in RNA, whereas heart, skeletal muscle, kidney and yolk sac had at least a 2-fold increase. This may reflect a difference in the enhancers used in these tissues, as liver and gut utilize two enhancers that lie 3' of the *H19* gene²¹, whereas the enhancers that act in mesoderm have not been identified. On the other hand, it may represent

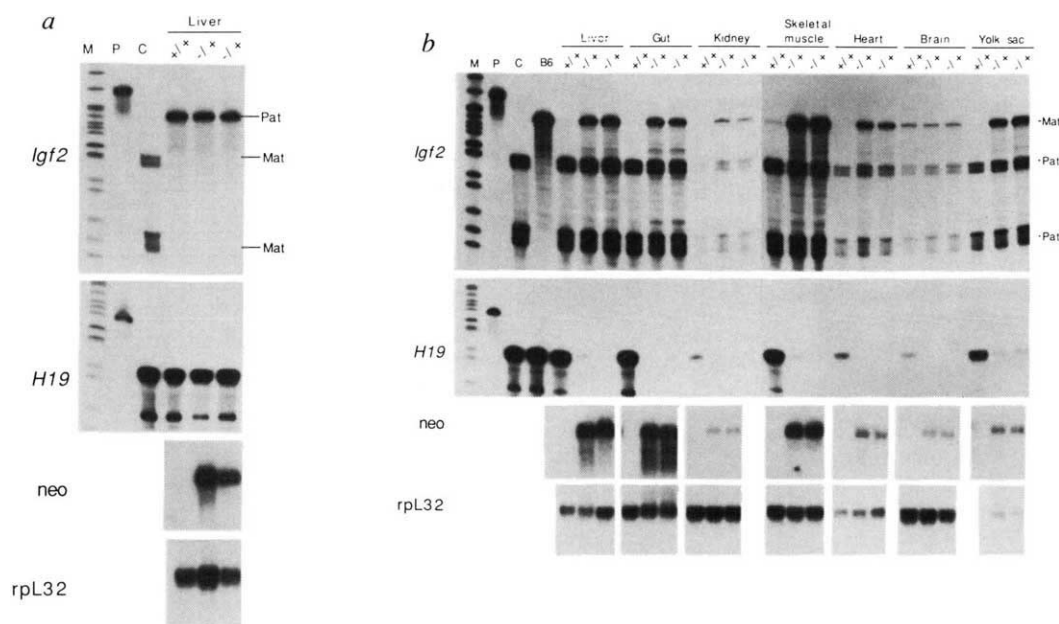


FIG. 2 Expression of the *H19* and *Igf2* genes in neonatal *H19* heterozygous mice. **a**, Paternal inheritance of the *H19* deletion. Heterozygous F_1 males were mated to B6(CAST-*H19*)¹⁸ females, and RNA was isolated from livers of neonatal pups. An allele-specific RNase protection assay that distinguishes between the 129Sv/Ev-derived paternal *Igf2* allele (Pat) and the *M. castaneus*-derived maternal allele (Mat) was used to assess the expression of *Igf2*. Lane designations are M, marker; P, undigested probe; C, *M. castaneus* RNA, wild-type (+/+) progeny RNA and heterozygous (+/-) progeny RNA. *H19* RNA was analysed using an RNase protection assay that does not distinguish between the two parental strains²¹. Expression of the *neo* gene and the control rpl32 gene were detected by northern blot analysis⁴⁷. **b**, Maternal inheritance of the *H19* deletion. Females heterozygous for the *H19* mutation were mated to males of the B6(CAST-*H19*) strain. The expression of *Igf2*, *H19*, *neo* and rpl32 RNAs were determined as in **a**. The B6 lane represents C57BL/6J-derived neonatal liver RNA, which behaves identically to 129Sv/Ev RNA. The maternal allele is 129Sv/Ev-derived (Mat) and the paternal allele is *M. castaneus*-derived (Pat). **METHODS.** Total RNA was isolated by LiCl-urea extraction⁴⁸. The amounts of RNA used in the *H19* and *Igf2* RNase protection assays and the ages of the animals were as follows: paternal inheritance, 5 μ g

of p2 liver RNA; maternal inheritance: p0 liver (5 μ g), kidney (2 μ g), skeletal muscle (3 μ g), and brain (10 μ g); e18.5 gut (5 μ g) and yolk sac (2 μ g), and p8 heart (3 μ g). *M. castaneus* and C57BL/6J p0 liver (5 μ g). p0 is the day of birth. The probe for the *Igf2* allele-specific RNase protection assay was obtained by amplifying with the PCR a 500-bp fragment of the 3' untranslated region of the *Igf2* gene using the forward primer 5'-CCACATCAGGCTGTTCC-3' and the reverse primer 5'-CTAGGGTCTGGGCTCTTC-3'. The product was cloned into the TA cloning vector (Invitrogen), linearized with *DraI* and used as a template for *in vitro* transcription by T7 polymerase in the presence of [³²P]CTP. The labelled RNA was hybridized to RNA overnight at 55 °C. Samples were incubated at 23 °C for 1 h in 20 mM Tris-HCl (pH 7.5), 600 mM NaCl, 10 mM EDTA, 80 μ g ml⁻¹ RNase A, 2 μ g ml⁻¹ RNase T1. The products were separated on a 7.5% acrylamide-7M urea gel and visualized by autoradiography. For northern analysis of *neo* and rpl32, 5 μ g total RNA was separated in a 1.5% agarose-6.3% formaldehyde gel, transferred to nitrocellulose and hybridized to a 630-bp *PstI*-*XbaI* fragment containing *neo*-coding sequences from the PGKneoBpA cassette⁴³. The blots were then stripped and reprobed with radiolabelled rpl32 (ref. 49) to control for differences in the amount of RNA in each sample.

tissue-specific epigenetic differences affecting the level of expression of the maternal *Igf2* gene. Nevertheless, PCR confirms that maternal deletion of the *H19* gene and its 5' flanking sequences results in an increase in maternal *Igf2* RNA in all tissues where the *Igf2* gene is imprinted.

The *Igf2* gene normally displays biallelic expression in only two tissues, the choroid plexus and leptomeninges of the brain⁷. When *Igf2* expression was examined in brains of maternal *H19* heterozygotes, both alleles were expressed at the same relative levels as in wild-type animals (Fig. 2b), although the amount of RNA increased in brain (Table 1). Thus the *H19* deletion has an effect on maternal *Igf2* expression only in those tissues in which *Igf2* is subject to imprinting.

In rodents, expression of the paternal *Igf2* allele ceases in most tissues by the fourth week after birth, with the exception of the choroid plexus and leptomeninges. After birth, the activated maternal allele of *Igf2* in the maternal *H19* heterozygotes was repressed in parallel with the paternal allele (Fig. 3), indicating that the two alleles were being regulated similarly. In the brain, where both *Igf2* alleles are equally expressed at moderate levels in the adult, the maternal allele in *cis* to the *H19* disruption was repressed to a slightly greater extent than the paternal allele, the only consequence of *H19* deletion in brain.

The *Ins-2* gene is paternally expressed in the visceral endoderm of the mouse yolk sac⁶, where *H19* and *Igf2* are also expressed. To test whether the imprinting of *Ins-2* is affected in yolk sac, maternal *H19* heterozygotes were crossed to a strain of mice containing the *M. spretus* alleles of the gene cluster in a BTBR background, BTBR(*M.spr-H19*). The alleles of *Ins-2* could be distinguished in e13.5 yolk sac by reverse-transcription PCR (RT-PCR) using a single-strand-conformation polymorphism (SSCP) in the *Ins-2* gene⁶. As in the case of *Igf2*, the deletion of the *H19* gene also disrupted the imprinting of *Ins-2*, as judged from the activation of the maternal allele in heterozygous animals (Fig. 4). The degree to which the maternal allele was activated, relative to the paternal allele, could not be accurately determined, because the combination of the reverse transcriptase and PCR reactions was biased in favour of the maternal allele, although this bias was much less evident upon amplification of genomic DNA (F1 lane in Fig. 4).

Paternal *H19* RNA expression in neonatal maternal heterozygotes was detected at a level of ~5% that of wild-type littermates, as judged by densitometry (Fig. 2b). This reflects the basal expression of the paternal *H19* allele, which occurs in wild-type animals as well, but is difficult to detect because of the overwhelming maternal *H19* signal (J. R. Saam and S.M.T.,

TABLE 1 Relative amounts of *Igf2* transcripts

Genotype	No. of samples	Tissue	RNA ratio $\pm$ s.e. (relative to wild type)
Cross I: <i>H19</i> ^{-/+} $\times$ <i>+/+</i>			
<i>H19</i> ^{-/+}	2	Heart	2.19 $\pm$ 0.004
<i>H19</i> ^{-/+}	2	Gut	1.39 $\pm$ 0.05
<i>H19</i> ^{-/+}	2	Yolk sac	2.14 $\pm$ 0.02
<i>H19</i> ^{-/+}	1	Liver	1.27
<i>H19</i> ^{-/+}	2	Muscle	1.98 $\pm$ 0.16
<i>H19</i> ^{-/+}	2	Kidney	2.11 $\pm$ 0.05
<i>H19</i> ^{-/+}	2	Brain	1.54 $\pm$ 0.08
Cross II: <i>H19</i> ^{-/+} $\times$ <i>+/+</i> <i>Igf2</i> ^{-/-}			
<i>H19</i> ^{-/+}	2	Liver	1.27 $\pm$ 0.07
<i>H19</i> ^{-/-} <i>Igf2</i> ^{-/-}	5	Liver	0.28 $\pm$ 0.007

The amounts of steady-state *Igf2* transcripts present in the samples of RNA in Fig. 2 were measured by a one-tube competitive RT-PCR assay⁴¹. For each sample, three different reactions were performed using serial dilutions of RNA in the presence of the same amount of competitor RNA, *rTth* polymerase (Perkin-Elmer), and the following primers: 5' ³²P end-labelled forward primer 5'-TCCTGTCTTCATCCTCTTCCAGCCCC-3', representing sequence of the non-coding exon derived from the third mouse *Igf2* promoter, and unlabelled reverse primer 5'-CGGTCCGAACAGACAACTGAAGCGT-3', representing sequence of the first coding exon. Competitor RNA was synthesized with Sp6 polymerase from a modified version of the *Igf2* sequence, cloned in plasmid pGEM-1 (Promega), after replacement of a 7/15 bp *KpnI*-*Bam*HI fragment with a 62/70 bp *KpnI*-*Bam*HI fragment from the poly-linker of plasmid pBSK(+) (Stratagene). The labelled DNA products from the *Igf2* and competitor RNA templates (199 and 254 bp, respectively) were fractionated on polyacrylamide gels and their radioactivity measured using a Phosphorimager. From these measurements, the amount of total RNA containing the same amount of *Igf2* transcripts was calculated as described⁴¹. The inverses of these values, representing *Igf2* amounts per unit of RNA assayed, were then corrected for relative amounts of RNA input, from the results of slot-blot analyses of serial RNA dilutions using a 28S ribosomal DNA probe. The averaged ratios mutant/wild-type of the final corrected values are shown here. A single wild-type value per tissue was used in cross I, whereas the average of four such values was used in cross II.

unpublished results). Unexpectedly, the *neo*^r gene on the maternal chromosome adopted the expression pattern of the host region, in that its tissue-specific and stage-specific expression was identical to that of *H19* (Fig. 2b). That is, its expression was highest in liver, gut and muscle, and lowest in kidney and heart, and was repressed in older animals (Fig. 3). Nevertheless maternal *neo*^r gene transcription did not preclude expression of *Igf2* or *Ins-2* in *cis*, demonstrating that expression of a gene *per se* at the *H19* locus is not sufficient to silence *Igf2* or *Ins-2*.

Growth and maternally inherited $\Delta H19$

A striking effect of the deletion of the *H19* gene in maternal heterozygotes at birth was an apparently uniform increase in body mass. At birth, maternal *H19* heterozygotes weighed 1.77 ± 0.12 g ($n = 12$), compared to 1.38 ± 0.12 g ($n = 13$) for their wild-type littermates, a 28% difference which persisted in adult mice as well, at least through to 4 months of age (Fig. 5). In so far as the maternal heterozygotes exhibit both a loss of function of *H19* RNA and a gain of function of *Igf2* and *Ins-2* expression, it was not possible to assign the cause of the somatic overgrowth to the effects of a single gene. In mice, insulin-like growth factor 2 is a potent fetal growth factor, as judged by the small size of animals with a deficiency in *Igf2*, making its overexpression a likely explanation for the overgrowth²². It is also possible that overexpression of *Ins-2* in the yolk sac contributes to the somatic overgrowth, although the function of *Ins-2* in the yolk sac is not clear, and its level of expression is low (data not shown). Finally, it has been shown in tissue culture cells that *H19* can function

as a tumour suppressor²³, and thus this non-coding RNA may have a role in growth control.

To test the role of *Igf2* in somatic overgrowth, heterozygous *H19* females were crossed to heterozygous *Igf2*-deficient males²². The doubly mutant animals should have reduced levels of *Igf2* relative to the *H19* single mutants, owing to the absence of paternal *Igf2*. As expected, animals mutant for *H19* alone (Fig. 5, squares) were larger, and animals mutant for *Igf2* alone (Fig. 5, triangles) were smaller than their wild-type littermates (Fig. 5, circles). Most important, the animals that were mutant for both genes (Fig. 5, diamonds) were indistinguishable in size from the wild-type animals in their birth weights and postnatal growth rates. Their normal body weight indicates that excess *Igf2* is necessary for the somatic overgrowth phenotype, and makes it unlikely that either overexpression of *Ins-2* or a lack of *H19* is a factor. Furthermore, the growth retardation of *Igf2* paternal heterozygotes and the overgrowth of *H19* maternal heterozygotes both occur exclusively during embryogenesis, whereas after birth both classes of mutants grow at the same rate as wild-type animals (Fig. 5, bottom). The *H19*^{-/+} heterozygous animals expressed 127% of wild-type levels of *Igf2* RNA in liver, whereas the doubly mutant animals expressed 28% of wild-type levels (Table 1). These findings highlight the reproducibility of the increase in *Igf2* in two different crosses, as well as the additivity of the paternal and maternal *Igf2* RNA levels.

No defect is apparent in *H19*^{-/-} *Igf2*^{-/-} mice, suggesting that, apart from its gain-of-function effects on *Igf2* and *Ins-2*, the loss of function of *H19* has no deleterious effect. To guarantee that the absence of an *H19*-specific phenotype was not due to the small amount of paternal *H19* RNA expression, homozygous *H19*^{-/-} animals were generated. At least until 3 months of age, they are fully viable and fertile, with somatic overgrowth being the only demonstrable defect (P.A.L., unpublished results).

Loss of imprinting of *Igf2* and *Ins-2*

The loss of imprinting of *Igf2* and *Ins-2* in the maternal *H19* heterozygotes establishes a causal relationship between sequences that lie within 10 kb of the 5' end of the *H19* gene, and/or the gene itself, and the imprinting of both *Ins-2* and *Igf2*, which are 95 and 80 kb further upstream, respectively. Thus one function of the *H19* region is to imprint its neighbouring genes. The *H19* disruption may be functionally equivalent to small deletions that have been detected in patients with Prader-Willi syndrome^{24,25}, a human genetic disorder that affects at least one imprinted gene. These deletions affect the expression and DNA methylation patterns of genes at least 300 kb away. Indeed, we find that in the presence of the maternal *H19* gene deletion, the upstream promoter region of the maternal *Igf2* gene adopts the methylation pattern of the wild-type paternal chromosome (P.A.L., data not shown).

This result fits a prediction of the enhancer competition model, that the removal of the *H19* promoter from the maternal chromosome should lead to the activation of the maternal *Igf2* gene. A second prediction of the model, that the genes use the same enhancers, has been met as well, based on the finding that deletion of two endoderm-specific enhancers that lie 3' to the *H19* gene²⁶ results in a loss of expression of *H19* on the maternal chromosome, and a loss of function of *Igf2* on the paternal chromosome in neonatal liver (P.A.L., J. R. Saam, C. Stewart and S.M.T., unpublished results). Further support has come from analysis of the human *IGF2* and *H19* genes, which are physically linked and imprinted in the same directions as in the mouse. In Wilms' tumour samples, however, where biallelic expression of *IGF2* has been observed, there is a concomitant silencing of *H19* expression, as required by the model^{27,28}.

If the expression of *Ins-2* is under the control of the same regulatory elements that govern *H19* and *Igf2* expression in yolk sac, its loss of imprinting can also be reconciled with the model. That is, the shared enhancers simultaneously activate *Igf2* and *Ins-2* when *H19* is in a silent state on the paternal chromosome.

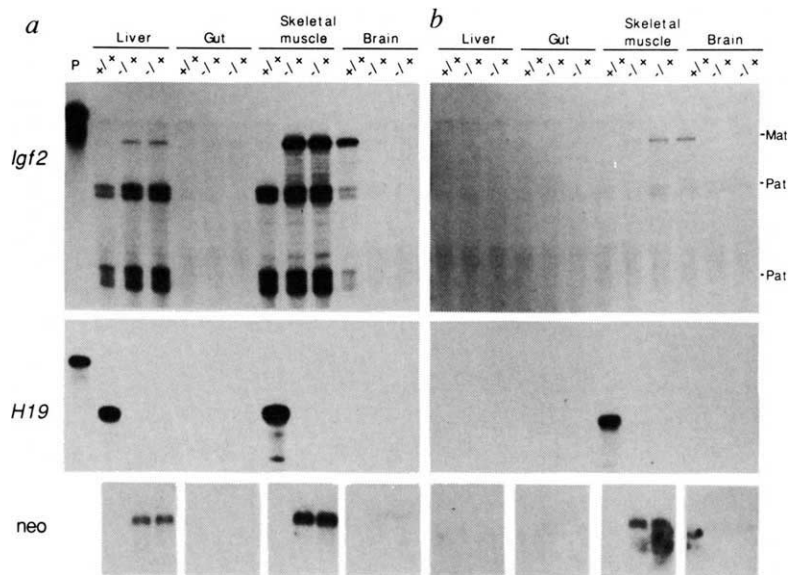


FIG. 3 Postnatal repression of *H19* and *Igf2* in *H19* heterozygous mice. Female heterozygous mice were mated to B6(CAST-*H19*) males and the offspring were killed at 2 weeks (a) and 4 weeks (b) after birth. RNA was prepared from liver, gut, skeletal muscle and brain, and the levels of *Igf2*, *H19*, *neo* and rpl32 RNAs were determined using 5 μ g of total RNA as for Fig. 2.

In pancreas, on the other hand, the β -cell-specific enhancers that lie 5' to the gene direct biallelic *Ins-2* expression^{29,30}. The fact that the impact of the deletion of the *H19* gene extends at least 95 kb to the *Ins-2* gene makes it plausible that *mash-2*, which is at least another 200 kb away, is also affected. This possibility is under investigation.

The *neo*^f gene behaved in the mutant mice as if it was under the control of *H19* regulatory elements, despite the fact that the *pgk-1* promoter is expressed ubiquitously²⁰. Whether this reflects the direct interaction between the *pgk-1* promoter and the *H19* enhancers, or is an indirect effect of the *neo*^f gene being in an 'open' chromatin domain, is not clear. In the first case, co-expression of *neo*^f and *Igf2* on both maternal and paternal chromosomes would apparently contradict enhancer competition. *Neo*^f expression can be reconciled with the model if the *neo*^f promoter is not as effective a competitor for the enhancers as the *H19* gene, and therefore all three genes are expressed on the same chromosome. The importance of relative promoter strength in enhancer competition has been documented for the

developmental switch between embryonic ϵ -globin and adult β -globin gene expression in chickens³¹. When the chicken adult β -globin promoter is slightly disabled by mutation, both that gene and the normally silent ϵ -globin gene are now expressed in the adult³², presumably because the competition between them is equalized.

What element(s) in the *H19* locus is essential for *Igf2* and *Ins-2* imprinting? The simplest answer is that there is a single imprint that acts on all three genes. We favour the idea that the initiating event is sperm-specific DNA methylation of the *H19* region, which is maintained after fertilization on the paternal chromosome. This inactivates the *H19* promoter and is both necessary and sufficient for the expression of *Igf2*. On the maternal chromosome, the strong activity of the unmodified *H19* promoter precludes *Igf2* expression. Another candidate for the imprint is a boundary element that blocks access of *Igf2* to the enhancers on the maternal chromosome, and is inactivated by DNA methylation on the paternal chromosome. Boundary elements such as the *scs* elements in the *hsp70* heat-shock gene³³ and

FIG. 4 The effect of the *H19* deletion on the imprinting of *Ins-2*. Female *H19* heterozygotes were mated to male BTBR(*M.spr-H19*) mice. Yolk sacs were dissected at e13.5, and RNAs from wild-type (+/+) and heterozygous (-/+) yolk sacs were transcribed by reverse transcriptase (+RT). A mock reverse transcription was performed for each sample in the absence of reverse transcriptase to control for DNA contamination (-RT). PCR amplification of the products of reverse transcription, together with genomic DNAs from C57BL/6J (*M. domesticus*), BTBR(*M.spr-H19*) (*M. spretus*) and (C57BL/6J $\times$ BTBR(*M.spr-H19*))F₁ animals was performed using oligonucleotide primers specific for *Ins-2* which reveal an SSCP between *M. domesticus* and *M. spretus*⁶. METHODS. RNA was isolated by extraction with guanidinium isothiocyanate and CsCl gradient centrifugation⁵⁰. The RNAs were treated with DNase I for 30 min at 37 °C before reverse transcription. The mixture was extracted with phenol:chloroform (1:1) and precipitated with 2 volumes of ethanol. Total RNA (10 μ g) was transcribed with reverse transcriptase for two 1-h cycles at 42 °C according to the directions with the cDNA Cycle Kit (Invitrogen) using oligo(dT) as the primer. PCR amplification of 100 ng genomic DNA or a portion of the cDNA reaction was done as described⁶ in the presence of [³²P]dCTP using 35 cycles of 95 °C for 30 s, 55 °C for 90 s, 72 °C for 90 s. PCR products were diluted in 95% formamide, denatured, and separated on a 0.5 $\times$ MDE gel (AT Biochem) for 6 h at 25 °C.

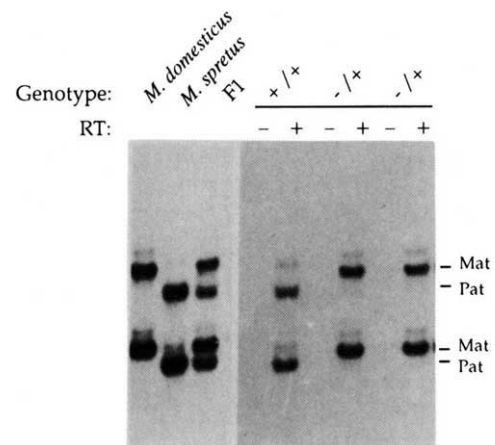
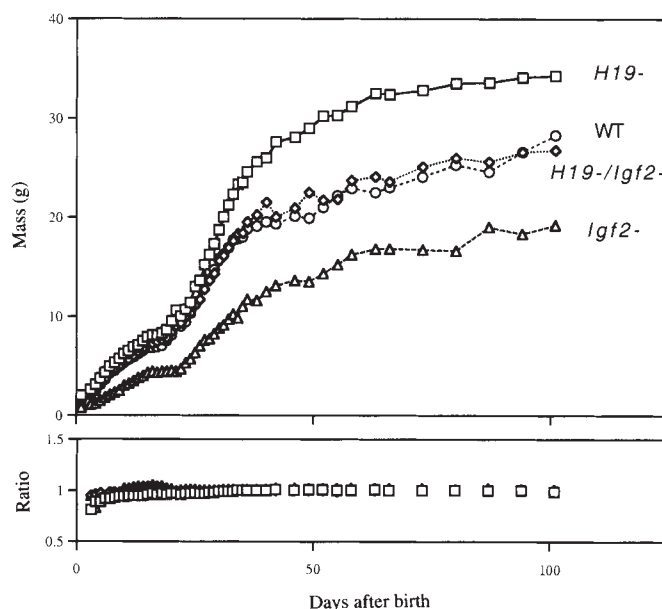


FIG. 5 Growth of progeny from the cross between heterozygous *H19* females and heterozygous *Igf2*-mutant males. Top, The masses in grams of 4 representative progeny in crosses between *H19*-/+ mothers $\times$ *Igf2*-/+ fathers were determined. The four progeny classes are indicated. Bottom, From the data in the top part, the percentage increase in weight at each time point was calculated as $(W_t - W_0) \times 100 / W_t$, where W_t is the weight at time t and W_0 is the weight at birth. The ratio of the per cent increase in weight for mutant and wild type was plotted versus time to show that the ratios do not deviate significantly from unity. Three symbols for the 3 mutant classes are those used in A. METHODS. Tail or toe DNA was prepared from 3-week-old pups. Primers specific for the *Igf2* deletion or the *H19* deletion were used in the PCR to genotype the animals. The *Igf2* primers were *Igf2*-forward primer 5'-CTAGCTCAAAGCCTGCGTTTCTTC-3' and *neo*' reverse primer 5'-TGCGCTGACAGCCGGAACAC-3'. The *H19*-specific primers, located in the bovine growth hormone gene polyadenylation signal sequences of the *neo*' cassette, were forward, 5'-CTAGAGCTCGCTGATCAGCCT-3', and reverse 5'-GAC-AGTGGGAGTGGCACCTT-3'.



suppressor of hairy wing elements in *Drosophila*³⁴ have been shown to block enhancer-promoter interactions when positioned between them. A single boundary element, however, fails to explain the silence of the paternal *H19* gene, unless enhancer competition is invoked in *Igf2*'s favour. Otherwise it is necessary to propose two independent marks on the paternal chromosome, one that inactivates the *H19* promoter and one that inactivates the boundary element. Finally, it is possible that the silencing of the maternal *Ins-2* and *Igf2* genes requires the product of the maternal *H19* gene itself. This proposal is derived from reports that the genes for untranslated RNAs that are similar in overall structure to *H19* have been described in two other imprinted loci.

Xist RNA is encoded by a gene that maps to the X chromosome inactivation centre and is exclusively expressed from the paternally inherited inactive X chromosome in early mouse embryos³⁵⁻³⁸. A non-coding RNA that maps to the Prader-Willi syndrome locus on human chromosome 15 is paternally expressed³⁹. The conservation of the primary and secondary structure in *Xist* and *H19* (refs 37, 40) argues that these genes serve some function in mammals, and their presence in imprinted domains suggests that they may function in initiating or maintaining the imprinting of neighbouring genes, a possibility that calls for other mutations to separate the transcriptional role of *H19* from its product. □

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An X-ray jet from the Vela pulsar

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THE well studied^{1–5} Vela pulsar, which has recently^{6,7} been demonstrated to lie at the centre of the Vela supernova remnant⁸, loses far more rotational energy than can be accounted for by radiation from the pulsar itself or from a surrounding, compact nebula^{9–11}. One theory suggests that the energy is carried away by an equatorial wind¹². Here we report the observation of an X-ray-emitting jet, which begins at the pulsar and extends roughly seven parsecs towards the south-southwest and along the pulsar spin axis¹³ (indicating a polar, rather than equatorial flow). This jet may provide the loss mechanism for most of the pulsar's rotational spin-down energy. One-sided jets, such as Vela, may also accelerate pulsars to large space velocities.

The Vela pulsar (PSR 0833–45) and its surrounding 1°-radius field were observed by the Rosat X-ray telescope with the Position Sensitive Proportional Counter (PSPC) at the focus during 1991 in two separate pointings: April 22.0–23.9 for 13,900 s and December 4.7–19.8 for 18,500 s. An image in the Rosat hard X-ray band (0.9–2.0 keV) of the combined pointings is shown in Fig. 1 and reveals a remarkable jet of diffuse emission which originates at the pulsar and extends to the SSW. This jet is 45 arcmin in length and 12 arcmin wide (full-width at half-maximum, FWHM), corresponding to 6.5 and 1.7 pc, respectively, at an assumed pulsar distance of 500 pc. The intensity profile across the jet feature appears to be gaussian and does not show any limb brightening. Diffuse X-ray emission has been detected previously from southwest of the pulsar⁹ by the Einstein satellite, but this Rosat observation is the first to reveal that the feature is narrow, collimated and remarkably symmetrical. We infer

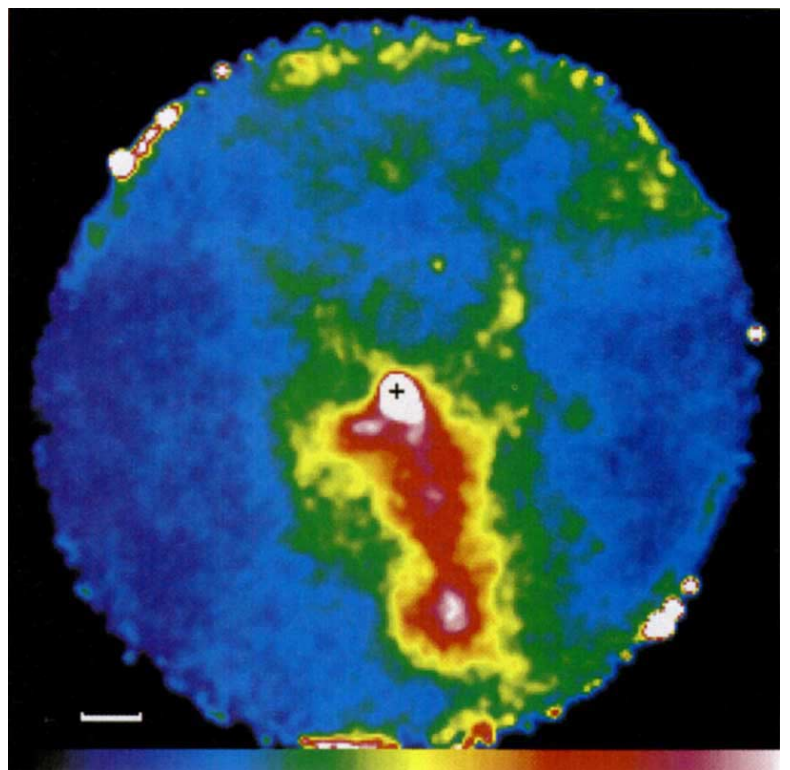
from the morphology of the feature and the fact that the pulsar lies at one end (at least in projection), that the feature is indeed a pulsar jet.

The spectrum of the jet was modelled either as emission from an optically thin thermal plasma, or as continuum emission with a power-law energy distribution. We have restricted our models to the energy range 0.7–2.4 keV—the range where the jet is visible—and have fixed the value of the interstellar neutral-hydrogen column density at $2.6 \times 10^{20} \text{ cm}^{-2}$ (ref. 9). The nebular X-ray background and its fluctuations were estimated from the entire PSPC field of view, excluding the region around the jet and pulsar. Although the spectrum contains >85,000 counts, fluctuations of ~15% due to the patchiness of the background are the dominant source of error, and limit the accuracy of the spectral fits. The resulting best-fit parameters are shown in Table 1. The values of χ^2 per degree of freedom were acceptable for both models. At an angular distance >5 arcmin (that is, beyond the compact nebula) there are no significant spectral variations over the jet's length. The total emitted luminosity in the 0.7–2.4 keV bandpass, for either model, is $\sim 10^{33} \text{ erg s}^{-1}$.

Even though both spectral models fit the data in the stated energy range, we prefer to adopt the thermal spectral model. A continuous power-law emission model does not accurately reproduce the data when extended to softer X-rays and even lower energies^{14,15}. It is conceivable that a more detailed model could be made consistent with the data, but it may be difficult to verify this hypothesis using Rosat because of its limited spectral resolution. In the rest of our discussion we assume that the X-rays observed from the jet are predominantly thermal emission from plasma with a temperature of 1.3 keV and a number density of 0.4 cm^{-3} .

The physical parameters of the jet such as its speed, mass flow and mechanical power can now be computed using existing models for extragalactic and protostellar jets^{16–19}. These models generally take the jet to be a beam of supersonic neutral material moving at speed v_j , which interacts with the ambient medium at a shock front often called a 'working surface' or 'head'. For the case of the Vela jet, the average speed of the head, v_h , can

FIG. 1 Rosat PSPC hard X-ray (0.9–2.0 keV) image of the Vela pulsar and a surrounding field of 1° radius. The pulsar and its 1-arcmin-radius compact nebula are at image centre (indicated by a cross). The jet projects from the pulsar towards the SSW (in this image, north is up and east is left). We also note that a weaker region of diffuse emission is present, which connects the pulsar to its apparent birthplace ~8 arcmin to the southeast, as determined from its age and proper motion¹⁴. The image has been corrected for telescope vignetting and is smoothed by a 45-arcsec-radius gaussian filter. Intensity levels range from 0 (black) to 9×10^{-4} (white) counts per second per pixel of 15×15 arcsec; see colour scale at bottom of figure. The horizontal white bar is 10 arcmin long.



be estimated directly (using the known pulsar age) to be at least $6.5 \text{ pc}/11,000 \text{ yr} \approx 570 \text{ km s}^{-1}$, and larger if the jet is inclined to our line of sight. Uncertainties in the pulsar distance and age could influence this value by a factor of ~ 2 . Behind the working surface, beam material which has been heated and deflected rearwards by the shock zones will collect in a 'cocoon' of hot gas²⁰. We propose that the observed X-rays are emission from the thermal plasma in the cocoon.

The speed of the jet can be estimated as follows, if we assume that the head moves with a constant velocity. A jet beam of density ρ_j acting on a working surface of area A_j exerts a force $A_j \rho_j (v_j - v_h)^2$, which must be balanced by the force of the ambient material, $A_h \rho_a v_h^2$, of density ρ_a impinging on a bow shock whose area is A_h . This leads to a solution for the jet velocity, $v_j/v_h = 1 + \sqrt{(A_h \rho_a / A_j \rho_j)}$. The term $A_j \rho_j$ can be eliminated from the equation by considering that the cocoon density must be made of ambient and jet-added components, or $\rho_c = \rho_a + (v_j/v_h)(A_j \rho_j)/A_c$. These equations can be combined, assuming that the areas of the head and cocoon are approximately equal and using density values from Table 1, to give the jet speed $v_j = 1.8 v_h \approx 1,100 \text{ km s}^{-1}$. Although this speed is non-relativistic, it is possible that the jet is ejected relativistically from the pulsar magnetosphere, but is slowed by entraining additional ambient material on its way to the working surface. $A_j \rho_j$ is $6 \times 10^{12} \text{ g cm}^{-1}$, the linear mass density in the jet. We estimate that these values are uncertain by a factor of 2–4.

The total mechanical power required to drive this jet is approximately the pulsar's rotational energy loss rate. The jet power is easily seen to be $\dot{E} = \frac{1}{2} \dot{M}_j v_j^2 = \frac{1}{2} A_j \rho_j v_j^3$, where $\dot{M}_j$ is the rate of mass flow in the jet. Using the solution above for $A_j \rho_j$ and v_j , we find that the jet must be powered by $4 \times 10^{36} \text{ erg s}^{-1}$, or almost 50% of the current pulsar spindown energy. Although this value is an order-of-magnitude estimate, it is remarkable that a direct measurement of the head velocity and the plasma parameters of the jet combine to yield a mechanical energy which is close to the correct value; this is a powerful argument for the pulsar-jet association.

As measured from the X-rays, the cocoon density exceeds the ambient density by a factor of four. This implies that there is a mass flow in the jet, at a rate of $\dot{M}_j = A_j \rho_j v_j \approx 10^{-5} M_\odot \text{ yr}^{-1}$. This is a huge mass flow which could not originate deep from within the pulsar magnetosphere, because it is energetically impossible to extract this much mass from the surface of a $1.4 M_\odot$ neutron star. Also, the pulsar itself could not have swept up this much mass via its northwesterly proper motion¹⁴. We suggest that the excess mass must come from the ambient medium by entrainment through the cocoon. Considering that the jet axis is nearly perpendicular to the direction of the pulsar proper motion, the jet may indeed be sweeping up ambient material all along its length. In fact, the Rosat image reveals that the jet curves slightly southwards, indicating that it may be interacting with the material and swept backwards.

Radio maps of the Vela region²¹ at a frequency of 408 MHz show that the end of the X-ray jet is coincident with Vela X, the brightest radio component of the supernova remnant, suggesting

that electrons, shock-accelerated by the sudden jet termination, are cooling by radio synchrotron emission. This X-ray connection between the pulsar and Vela X was first noticed by Harnden *et al.*⁹ in 1985, but at that time the cause of the X-rays was unclear because the pulsar's proper motion was not established. Radio polarization measurements of the same region²² show that the projected magnetic fields are generally aligned along the X-ray jet, bringing the possibility that the cocoon, and perhaps the jet itself, are magnetically confined.

There are several interesting implications of pulsars with jets, beyond simple novelty. First, a jet provides a clear way for the pulsar to re-energize its supernova remnant over the pulsar's lifetime, as the pulsar can deposit its total 'birth' rotational energy, $E_{\text{rot}} \approx 10^{49} - 10^{51} \text{ erg}$, comparable to the total mechanical energy in a supernova explosion. Thus it may be possible for jets to alter the shape of supernova remnants over a long time period; Manchester²³ has pointed out that many supernova remnants appear to have a 'biconical' configuration associated with a bipolar pulsar outflow.

A second consequence of pulsar jets is that asymmetric jets can accelerate the neutron star after the supernova explosion. Assuming no counterjet, the force on the Vela pulsar is $A_j \rho_j v_j^2 \approx 8 \times 10^{28} \text{ dyn}$. Over the 10^4 -yr pulsar lifetime²⁴, this force could impart a velocity of 90 km s^{-1} which is comparable to the pulsar's current transverse proper motion¹⁵ of $\sim 110 \text{ km s}^{-1}$, but not in the correct direction. If similar conditions hold for the next 10^5 yr , Vela could be transformed from a slow- to a fast-moving pulsar. Although it does not appear that the jet is entirely responsible for Vela's present proper motion because the directions at the proper motion and the jet are not aligned, jet acceleration does provide an intriguing alternative to the supernova explosion 'kick' theories often invoked to explain proper motions. The remarkable fact that the observed mean pulsar velocity²⁵ is 450 km s^{-1} may be a consequence of post-supernova acceleration mechanisms such as one-sided jets, rather than pulsars being born with high velocities.

There remains the question of the one-sidedness of the Vela pulsar jet. Because relativistic effects are excluded for the velocities we derive here, we can only suspect that either there must be asymmetries involved in the pulsar magnetic field making the jet one-sided; or alternatively, the effect of anisotropies in the distribution of material around the pulsar may modify the counterjet beyond recognition.

We must stress that the jet model we describe here is not unique. For example, we have not examined the synchrotron model in depth, as it did not seem consistent with observations at other wavelengths. Additionally, our thermal plasma spectral modelling has assumed a uniform plasma density; the effects of clumping, for instance, would reduce average densities and introduce multitemperature components. These are configurations which cannot be distinguished by Rosat. We anticipate that future multiwavelength measurements, together with improved instrumentation and more sophisticated modelling, will fully determine the nature of the Vela jet. $\square$

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TABLE 1 Results of spectral models for Vela pulsar region

Angular distance (arcmin)	Plasma			Power law	
	Temp. (keV)	Density (cm^{-3})	Pressure ($10^{-8} \text{ erg cm}^{-3}$)	L_x (0.7–2.4 keV) ($10^{32} \text{ erg s}^{-1}$)	α
2–5	6.7	1.2	2.5	0.9	1.7
5–45	1.3	0.4	0.19	7.0	3.1
Ambient	0.12	0.11	0.004	—	—

Temperature, density and pressure of Vela's compact nebula (which dominates in the 2–5 arcmin region), the jet (5–45 arcmin), and the ambient supernova remnant. We assume that the depth of the jet in the line of sight is $\sim 1 \text{ pc}$ for density calculations, and that the distance to the pulsar is 500 pc to determine luminosity L_x . The parameter α is the index of the incident photon number spectrum. Formal statistical errors are $\sim \pm 20\%$ at a 2σ confidence level.

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Magnetic reconnection as the origin of X-ray jets and H α surges on the Sun

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THE solar corona (the outermost portion of the Sun's atmosphere) is far hotter than the 'surface' (the photosphere). Recent observations of X-ray jets^{1–4} (collimated flows of plasma at temperatures of a few million degrees) suggest that magnetic reconnection—the cutting of stressed magnetic field lines, which is associated with a violent release of energy, and their subsequent reconnection—may be responsible for heating the corona⁵. But the physical relationship between the X-ray jets, microflares (localized impulsive bursts whose total energy is below the level of the standard flares) and cooler H α surges⁶ (jets of gas at a temperature of about 10,000 K) has been unclear. In particular, it has been thought⁷ that H α surges and X-ray jets must arise from independent processes, on the grounds that reconnection would heat any plasma to X-ray-emitting temperatures. Here we present the results of magnetohydrodynamic simulations of the reconnection process, which show that X-ray jets and H α surges can be ejected simultaneously from microflares^{8,9}. This suggests that the total energy associated with the microflares is much greater than previously thought, and may be significant in heating the corona.

Many solar X-ray jets are known to be ejected from emerging flux regions^{1–3}. This suggests that the magnetic energy of the emerging flux¹⁰ is converted to the bulk kinetic and internal energy of the jets. How can this conversion take place? The model of Shibata *et al.*¹¹ is as follows: two separate magnetic field lines of emerging flux and of pre-existing coronal field come close together due to the rising motion of the emerging flux. By the effect of finite resistivity, they are cut and are reconnected with each other. The reconnection disconnects some of the highly stressed field lines in the emerging flux region and causes these field lines to fly outwards at one end as they try to straighten out. This whip-like motion accelerates (like a sling-shot) the plasma that lies on these field lines. At the same time, by Joule dissipation, magnetic energy is partly released as heat to increase the temperature of the plasma to a level when it emits X-rays. As a result, hot plasma is ejected from the reconnecting region, and may be observed as the X-ray jets.

Figures 1 and 2 show the results of numerical simulations of this model. The simulations are performed by solving the two-dimensional resistive magnetohydrodynamic (MHD) equations with uniform gravitational field. (See Shibata *et al.*⁸ for detailed information.) For heat loss/gain, the effect of heat conduction and radiative cooling is neglected. The initial state of the simulation is a hydrostatic plasma layer of three regions: corona (10^6 K), chromosphere/photosphere (10^4 K), and convection zone (with convectively unstable stratification). Initially, the interface of corona and chromosphere is interpolated smoothly by the hyperbolic-tangent function. To simulate the emerging flux, we put a horizontal magnetic flux sheet in the convection zone. The emergence of the flux sheet is reproduced by simulat-

ing the magnetic buoyancy instability^{12–14} (the Parker instability). To excite this instability, a small perturbation is initially imposed on the magnetic flux sheet within a finite horizontal domain. As for an initial coronal field, we studied two cases; (1) a uniform oblique field (with inclination angle of 45° ; Fig. 1), and (2) an approximately uniform horizontal field (Fig. 2). Note that, in case (1), although there is initially a kink at the transition between the horizontal magnetic flux sheet and the oblique coronal field (Fig. 1), it has little effect on the dynamics because it is embedded in a dense gas which is not moved by the effect of the kink on the timescale of the simulations. The plasma is assumed to have anomalous resistivity^{15–17}, whose functional form is taken to be $\eta=0$ for $v_d < v_c$ and $\eta \propto (v_d/v_c - 1)^2$ for $v_d \geq v_c$, where $v_d \equiv J/\rho$, is the non-dimensional (relative ion-electron) drift velocity, ρ is the non-dimen-

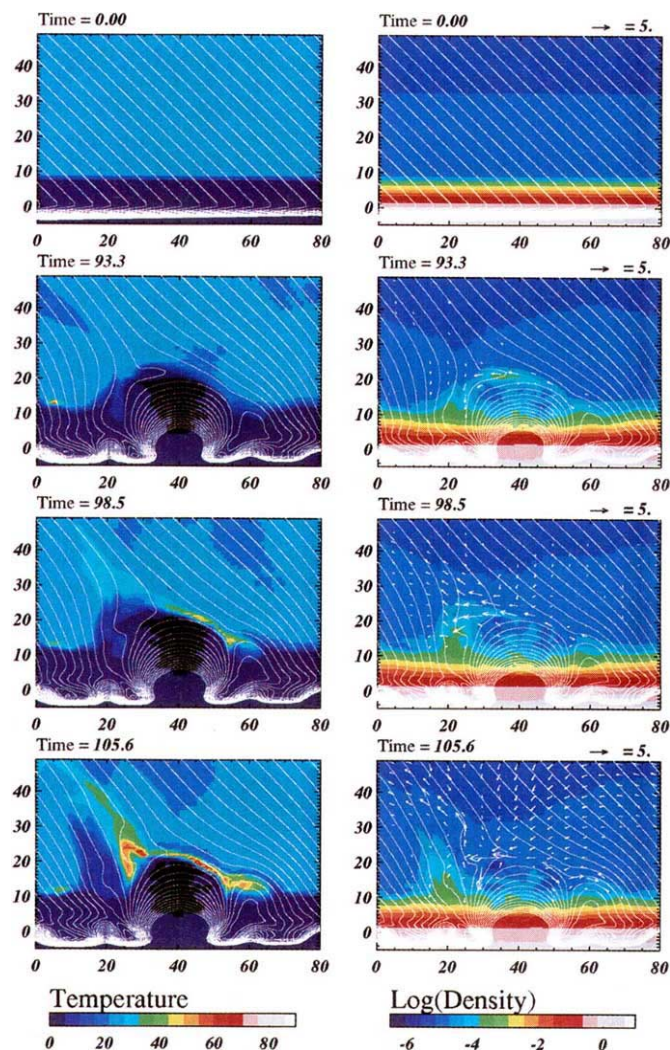
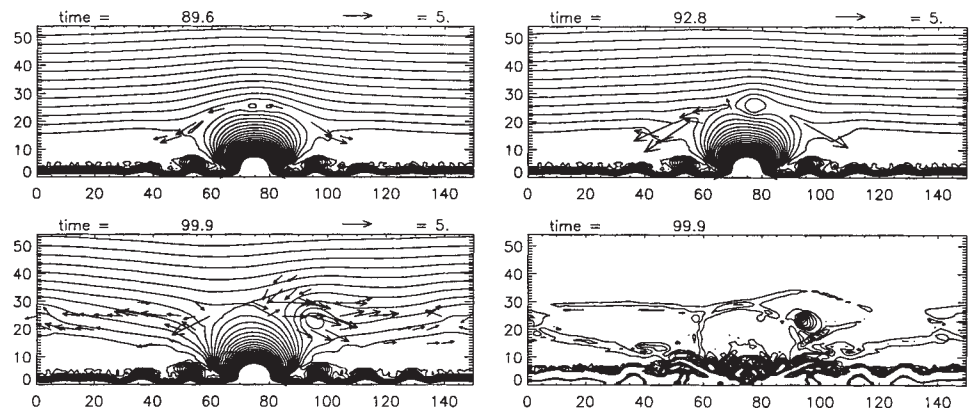


FIG. 1 Simulation of the reconnection of oblique field. The left-hand column shows temperature (T ; colour map) in units of 10^4 K and magnetic field lines ($\mathbf{B}$; lines). The right-hand column shows density ($\log_{10} \rho$; colour map) in units of $10^{-7} \text{ g cm}^{-3}$, magnetic field lines ($\mathbf{B}$; lines), and velocity vectors ($\mathbf{v}$; arrows). The times are in units of $\tau = H/C_s \approx 20$ s, and the length is in units of $H \approx 200$ km, where H and C_s are pressure scale height and sound speed in the photosphere/chromosphere, respectively. The parameters used in this simulation are as follows; initial coronal and chromospheric/photospheric temperature is 25 (which is somewhat low, but which has a weak effect on the result) and unity; vertical range of chromosphere/photosphere is 8 ($\approx 1,600$ km); thickness of the chromosphere–corona interface is 0.5 (≈ 100 km); plasma β (that is, ratio of gas pressure p to magnetic pressure $B^2/(8\pi)$) in the corona and in the horizontal magnetic sheet in the convection zone is 0.2 and 4.0, respectively; typical magnetic Reynolds number at the location of reconnection is ~ 300 .

FIG. 2 Simulation of the reconnection of horizontal field. Panels show magnetic field lines $\mathbf{B}$ and velocity vectors $\mathbf{v}$ for $t=89.6, 92.8, 99.9$ and current density J_y at $t=99.9$. The units of times and lengths are the same as in Fig. 1. The scale of velocity vectors is shown as an arrow at the top right of each figure, whose length indicates 5 in units of C_s .



sional mass density, J is the current density, and v_c is threshold above which anomalous resistivity sets in. The numerical code we use here has been thoroughly examined, and has been used for the study of the two-dimensional evolution of the Parker instability by previous authors¹²⁻¹⁴.

In the simulation, as expected, the magnetic loops expand by the Parker instability (Fig. 1). The distance between the two footpoints of the expanding loops is $\sim 4,000$ km. When the top of the rising loops make contact with the coronal field, reconnection takes place between the two fields: the plasma at the interface is heated by Joule dissipation to X-ray temperature (~ 4 – 10 MK). At the same time, the field lines of emerging flux release their stress by straightening themselves out, accelerating hot plasma and creating a pair of hot jets (Fig. 1). One of the pair is ejected upward. The velocity of the hot jet reaches ~ 100 km s^{-1} ,

(which is approximately the Alfvén speed that is realized when the magnetic energy is converted to the bulk kinetic energy) and the total bulk kinetic energy is $\sim 1.2 \times 10^{28}$ erg. These values are in good agreement with the typical observations of X-ray jets. Another interesting feature in this simulation is the high-temperature loops at the right side of the emerging flux in Fig. 1. This is the other product of the reconnection: while one of the pair of jets is ejected upward, the other goes down, colliding with the loops at the side of the emerging loops, and being compressed strongly there to form a very hot loop. This hot loop may account for some observations of a bright point (maybe unresolved bright loops) slightly away from the footpoint of the X-ray jets^{2,3}.

In addition to these hot features, it is important that our simulation also reveals the whip-like motion of cool plasma (Fig. 1). The cool plasma in this motion is originally from the chromosphere, which is carried up with the expanding loops, ejected by the sling-shot effect (that is, acceleration by the tension force of disconnected field lines) due to the reconnection, and finally shows the whip-like motion. The velocity of this whip-like motion is a few tens of kilometres per second, and it may be observed as a cool jet, namely an $H\alpha$ surge.

Figure 2 shows the results of our simulations in the case of horizontal field. In this case, multiple magnetic islands are created in the current sheet. Each island confines cool, dense, and high-pressure chromospheric plasma originally in the emerging loop. Soon after creation, the islands coalesce to a single large island (Fig. 2; $t=92.8$), and the resulting large, cool island is ejected horizontally out of the current sheet. At the same time, the plasma near the neutral point is heated to X-ray-emitting high temperature, and is ejected to both sides of the neutral point, forming a pair of horizontal hot jets. The velocity of the jets is ~ 100 km s^{-1} . Though the geometry and dynamics in the horizontal-field case are somewhat different from those in the oblique-field case, we have found some common physical processes, such as the ejection of a cool jet (or blobs) and a pair of hot jets.

What are actual observations of X-ray jets on the Sun? Shibata *et al.*¹¹ found two types of interaction between emerging flux and coronal field, which result in ejection of X-ray jets. One is the anemone-jet type¹⁸ (Fig. 3a), which occurs when emerging flux appears in a coronal hole where magnetic field is vertical or oblique (Fig. 3c). The other is the two-sided-loop type (Fig. 3b), which occurs when emerging flux appears in a quiet region where magnetic field is almost horizontal. Hot plasma is ejected along the coronal loops away from both sides of the emerging flux (Fig. 3d). The simulations shown in Figs 1 and 2 explain quite well these two observed types of interaction and their associated jets and loops. That is, the oblique-field case corresponds to the anemone-jet type, and the horizontal-field case corresponds to the two-sided-loop type.

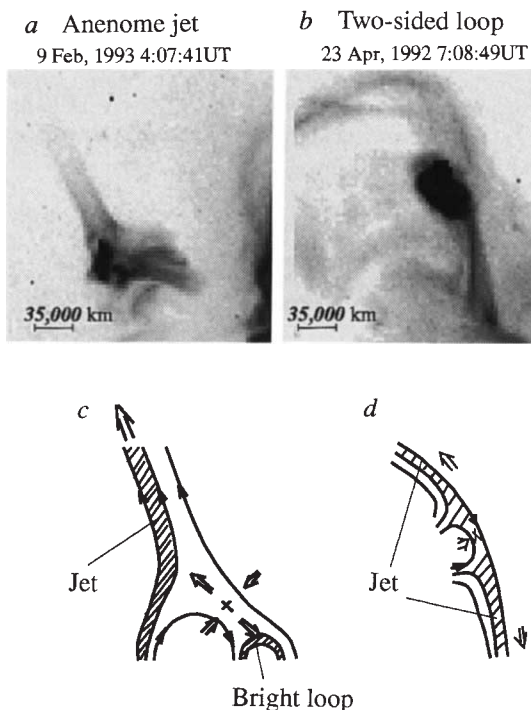


FIG. 3 Examples of two types of solar coronal X-ray jets observed by the Yohkoh soft X-ray telescope (after Shibata *et al.*¹¹). In the anemone-jet (a), the active region at the footpoint of the jet looks like a sea-anemone. In the two-sided-loop type (b), a pair of jets appears at both sides of emerging flux along the nearly horizontal field. c and d, Are schematic pictures of models for each type (after Shibata *et al.*¹¹).

It has long been thought that the cool $H\alpha$ surges could not be explained by magnetic reconnection because reconnection would heat any cool plasma to X-ray temperatures. Consequently, it has generally been argued that surges require separate mechanism which accelerates the plasma without heating it at the same time. Our simulations now provide a possible physical picture of how magnetic reconnection can accelerate cool plasma ($H\alpha$ surges) without much heating it. Our simulations also explain the coexistence of X-ray jets with $H\alpha$ surges observed in some cases (ref. 1 and R. C. Canfield *et al.*, manuscript in preparation). Note also that our simulation has predicted a spatial offset of the $H\alpha$ and X-ray jets, recently observed by Canfield *et al.* (manuscript in preparation). The cool magnetic island that is ejected horizontally in the horizontal-field case has not yet been observed, probably because of difficulties in resolving such fine structure. It will be interesting to search in future observations with high spatial resolution for ejection of this cool island.

Recently, Shimizu *et al.*¹⁹ discovered many transient brightenings in active regions with the Yohkoh²⁰ soft X-ray telescope²¹. These transient brightenings correspond to spatially resolved microflares which have been often thought to be one of main mechanisms of the coronal heating⁵. Although the origin of these transient brightenings is still a puzzle, there is a possibility that they are the result of reconnections similar to those discussed above. The horizontal jets of the two-sided-loop case are not necessarily observed as jets, and instead they are more likely to be observed as transient loop brightenings if the horizontal field is a part of a coronal loop. Thus, our results may suggest that the jets and the loop brightenings are not physically independent but closely related. In relation to the coronal heating, it has been found that observed microflares do not possess enough energy to account for the coronal heating²². But the common physical origin of jets and loop brightenings strongly suggests the possibility of association of these two features, and hence that the relevant volume and the total energy of the jets and loop brightenings may be larger than those inferred from observations of loop brightenings. This suggests also that the estimate of the total energy in each loop brightening might be an underestimate.

The numerical simulations presented here should help the understanding of the physical relation between jets (both X-ray and $H\alpha$), loop brightenings and coronal heating, and further suggests the importance of magnetic reconnection^{23,24} in solar coronal activity. $\square$

Prediction of ligand-promoted dissolution rates from the reactivities of aqueous complexes

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EARTH scientists have long recognized^{1–4} that the soluble organic acids excreted by soil biota enhance rates of mineral weathering, thereby chemically stratifying the soil and affecting the biodegradation pathways of organic matter, including pollutants⁵. Multidentate organic ligands^{6,7} also exist in industrial waste waters⁸ and can enhance the mobility of heavy elements, including radionuclides⁹. Here we examine whether rate coefficients for ligand-promoted dissolution of minerals can be predicted from existing studies of dissolved metal complexes. We have performed dissolution experiments on bunsenite (NiO) to compare with published studies of ligand exchange around dissolved Ni(II)-ligand complexes^{10–12}. The hypothesis is confirmed with surprising detail: the dissolution rate coefficient increases with the number of ligand functional groups coordinated to the surface metal, as do the exchange rate coefficients^{10–12}. Furthermore, we find that the dissolution rate coefficients can be predicted from the equilibrium constants for metal complexation in solution, indicating that the activated surface complexes resemble the corresponding dissolved complexes in important ways.

Furrer and Stumm¹³ established a framework for treating the dissolution of oxide minerals by showing that rates are proportional to the concentration of adsorbed protons and surface metal-ligand complexes. These adsorbates enhance the reactivity of the mineral surface and, to a suitable level of accuracy, proton- and ligand-promoted pathways can be treated as independent. For pH less than the point of zero charge, and with only a single rate-enhancing ligand complex at the surface, a suitable rate law is¹³:

$$\text{Rate (mol m}^{-2} \text{ s}^{-1}) = k_{\text{H}^+} [\text{H}^+]^n + k_{\text{L}} [\text{L}]^n \quad (1)$$

where k_{H^+} and k_{L} are the rate coefficients for dissolution via the proton-promoted and ligand-promoted pathways, respectively. The variables $[\text{H}^+]$ and $[\text{L}]$ are concentrations of the surface complexes and n is a rate order. The structure of metal-ligand surface complexes is important. Organic ligands that form bidentate rings are more effective than monodentate ligands in promoting dissolution, and smaller (five- and six-membered) bidentate rings are much more effective in enhancing rates than larger rings^{13,14}.

We can extend these results to yield information about surface reaction mechanisms if there is close correspondence between the ligand-exchange reaction in solution and metal removal from a dissolving surface. One observation^{10–12} from ¹⁷O-NMR is that the reactivity of bonds between metals and hydration water molecules increases with the number of certain other ligands in the inner coordination sphere (Fig. 1a). Carboxylates, amines and some inorganic ligands enhance the rates of water exchange by reducing the metal charge and weakening bonds to distal oxygens¹⁵. When translated to a dissolving mineral, the observation suggests that rate coefficients for dissolution increase predictably with the number of similar functional groups coordinated to a surface Ni(II).

An additional, powerful, observation is that the rate coefficients for exchange of many of these ligands correlate with

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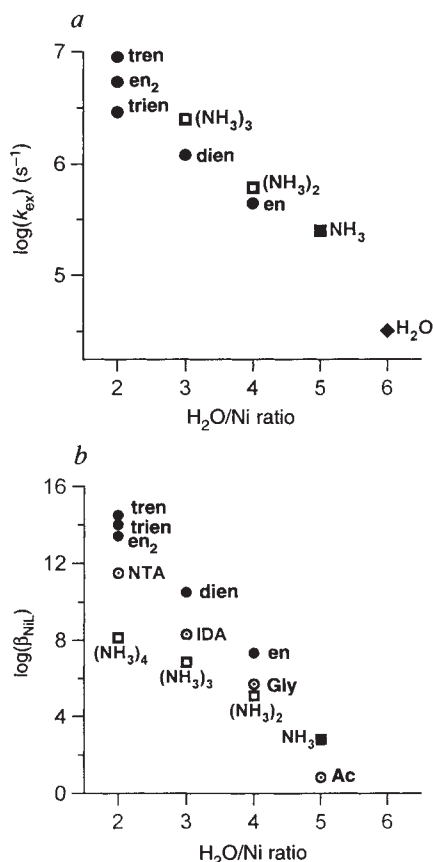


FIG. 1 a, First-order rate coefficients^{10,11} at 298.2 K for exchange of water between the bulk aqueous solution and water molecules in the inner-coordination sphere of Ni(II)-ligand complexes. The total coordination number of the metal (water molecules + coordinated atoms from the ligands) is six for all of the complexes shown. The ammonia complexes are identified according to the number of ammonia coordinated to the Ni(II). The label $(NH_3)_2$, for example, corresponds data for the $Ni(H_2O)_4(NH_3)_2^{2+}(aq)$ complex. The ligand abbreviations are: en, ethylenediamine $(NH_2(CH_2)_2NH_2)$; dien, diethylenetriamine $(NH_2(CH_2)_2NH(CH_2)_2NH_2)$; trien, $NH_2(CH_2)_2NH(CH_2)_2NH_2(CH_2)_2NH_2$; tren, $N((CH_2)_2NH_2)_3$. The point marked $\blacklozenge$ is the rate of solvent exchange around $Ni(H_2O)_6^{2+}(aq)$. Symbols are assigned to complexes in sets of homologous ligands ($\square$, ammonia complexes; $\bullet$, aliphatic amine complexes). The complex denoted $\blacksquare$ corresponds to the $Ni(H_2O)_5NH_3^{2+}(aq)$ complex, which can be assigned to both sets of homologous ligands. b, Metal-ligand stability¹⁶ constants for equilibrium in the reaction $Ni^{2+}(aq) + pL^{q-}(aq) = NiL_p^{2-pq}(aq)$ increase as a function of the $H_2O/Ni(II)$ ratio in the complex for ammonia ($\square$), amine ($\bullet$) and mixed amino-carboxylate ($\circ$) complexes. The stability constants correspond to an ionic strength of 0.1 M (2 M for $NH_3(aq)$) and a temperature of 298.2 K.

the equilibrium constants for complexation (Fig. 1b). This correlation (compare Fig. 1a,b) suggests that rate coefficients for mineral corrosion by ligands with similar functional groups can be predicted from tabulated data and only a few experiments.

To test these two hypotheses we determined rate coefficients for mineral dissolution in the presence of ligands that have increasing numbers of rate-enhancing functional groups, but for which the ring sizes are constant. Coordination of a surface Ni(II) to one amino and one carboxyl group of the ligands (Gly, NTA, IDA) shown in Fig. 2 forms a five-membered ring regard-

less of the molecule size. Rings between Ni(II) and paired carboxyl groups in these ligands are not favoured and acetate has only a single carboxyl functional group, and therefore forms a monodentate complex at the mineral surface. At our experimental conditions, all of these ligands form strong complexes with Ni(II) in solution¹⁶ and at the NiO(s) mineral surface.

Dissolution experiments were conducted using the pH-stat method¹⁷ under anoxic conditions and with apparatus that was shielded from light. Dissolution rates were estimated from the fluxes of Ni(II) which became constant (Fig. 2) after the earlier

FIG. 2 Concentrations of dissolved Ni(II) in the experimental solutions as a function of time. The concentrations are normalized to the mineral surface area used in each experiment, so that the slope of the data indicates the specific dissolution rate ($\text{mol m}^{-2} \text{s}^{-1}$). The lines indicate the data used to estimate the rates. The structures of the ligands are shown in shorthand notation adjacent to the corresponding concentration data. Error bars correspond to the precision of replicate experiments or to the analytical uncertainties, whichever is larger. Experiments were conducted by dispersing NiO(s) (with a surface area of $4.56 \text{ m}^2 \text{ g}^{-1}$ as determined by the BET method) in a stirred, isothermal (298.2 K) reaction vessel (0.125 l), corresponding to suspension densities of $7.5\text{--}30 \text{ g l}^{-1}$. Ligand concentrations were maintained at 0.010 M and the solution ionic strength was constant at 0.10 M (Na)ClO₄(aq). Determinations of Ni(II) concentrations were by atomic-absorption spectroscopy.

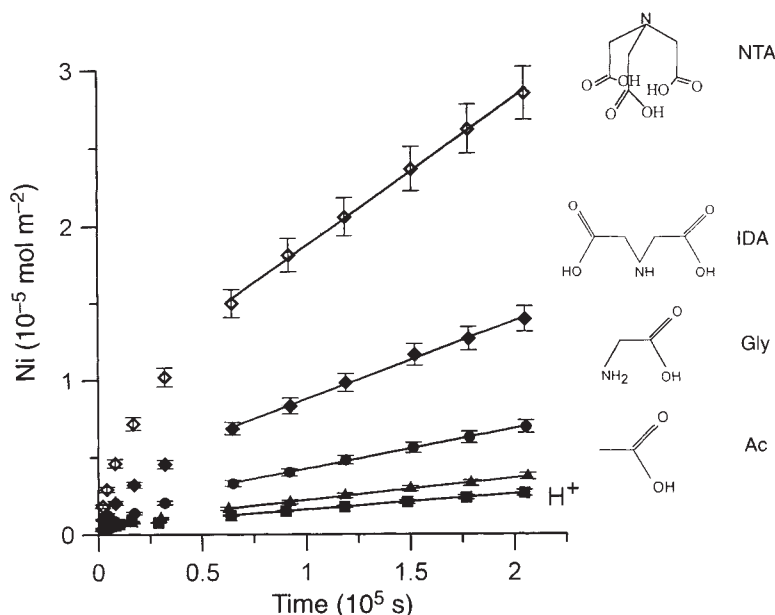


TABLE 1 Rates R_L and rate coefficients k_L for the ligand-induced dissolution of bunsenite

Ligand	R_L pH=6.0 (mol m ⁻² s ⁻¹)	log (k_L) pH=6.0 (s ⁻¹)	R_L pH=8.5 (mol m ⁻² s ⁻¹)	log (k_L) pH=8.5 (s ⁻¹)	log β_{NiL}^*
NTA	$87.4 (1.1) \times 10^{-12}$	-4.58 (0.06)	$20.0 (1.8) \times 10^{-11}$	-4.15 (0.04)	11.50
IDA	$42.5 (5.3) \times 10^{-12}$	-4.84 (0.06)	$10.3 (1.0) \times 10^{-11}$	-4.53 (0.05)	8.30
Gly	$18.2 (2.8) \times 10^{-12}$	-5.11 (0.07)	$2.9 (0.3) \times 10^{-11}$	-5.17 (0.05)	5.78
Ac	$6.6 (1.7) \times 10^{-12}$	-5.57 (0.13)	$4.9 (2.5) \times 10^{-13}$	—	0.84

The rate coefficients k_L were calculated by evaluating equation (1) using the rates for the proton-promoted dissolution R_{H^+} measured as $7.6 (0.9) \times 10^{-12}$ and $4.1 (2.0) \times 10^{-14}$ (mol m⁻² s⁻¹) for pH values 6 and 8.5, respectively. The value k_L for the ligand Ac could not be estimated because the amount of Ac adsorbed onto NiO(s) was too small. Standard deviations are in brackets.

* Stability constants¹⁶ for the nickel-ligand complex formation at 298.2 K and an ionic strength of 0.1 M.

stages of an experiment. Solids were separated from the solution after an experiment by vacuum filtration and the amounts of adsorbed carbon and nitrogen were measured using a Fisons carbon/nitrogen analyser. The rate coefficients for ligand-promoted dissolution were derived by evaluating equation (1) with the adsorbed ligand concentration, the bulk dissolution rate, and the relatively small rates of proton-promoted dissolution (Table 1).

We observe that the rate coefficients for ligand-promoted dissolution increase substantially with the number of coordinating functional groups (Fig. 3a). The simplest explanation is that the ligand functional groups displace surface hydroxyl groups or water molecules from around a surface Ni(II) site to form an inner-sphere surface complex^{13,18}, in which one surface Ni(II) bonds directly to the electron-donating atoms of the ligand. With this explanation, the mechanisms of ligand-promoted dissolution

can be directly compared with the dissolved metal-ligand complexes (Fig. 1a).

Most importantly, the magnitude of the effects is similar in both ligand-promoted dissolution and for water exchange around the dissolved complexes. The ligand NTA, which is the most effective of the studied ligands, increases^{10,11} the rates of water exchange in dissolved Ni(II) complexes by a factor of $\approx 10^{1.5}$. This increase is approximately equal to that observed for the corresponding ligand-promoted dissolution rate coefficients (Fig. 3a, Table 1), indicating similarities in the activated states.

Our second hypothesis, that the rate coefficients for ligand-promoted dissolution correlate with equilibrium constants, is also confirmed. In the absence of intrinsic equilibrium constants for adsorption, we correlate the dissolution rate coefficients with the stability constants for the corresponding dissolved complex (Fig. 3b). The resulting positive correlation (Fig. 3b) demonstrates that rate coefficients for ligand-promoted dissolution can be predicted from the solute stability constants. The correlation exists because the activated complex at the mineral surface resembles the dissolved metal-ligand complex. This similarity was suggested by Furrer and Stumm¹³ from the results of proton-promoted rates. They maintained that detachment from the mineral surface of a nearly formed metal complex is the rate-controlling step in dissolution. We now extend this conclusion to ligand-promoted reactions and show that the detailed spectroscopy of dissolved metal-ligand complexes can be used to infer structural characteristics of surface and activated complexes.

The pH is an important complicating factor because surface charge can oppose the charge on functional groups and because adsorbed ligand complexes can be protonated differently from complexes in the aqueous solution¹⁸. Such a difference in protonation may account for the observed variation in the dissolution rate coefficients for the higher-molecular-weight ligands (NTA, IDA) with pH (Table 1). We interpret the variation to result from partial protonation of the surface-ligand complexes at pH 6, which decreases the reactivity. The larger ligands exhibit this effect because they have more sites to be protonated in a surface complex. To account for this variation, dissolution could be treated as the sum of two surface-ligand complexes that differ in the extent of protonation and reactivity with pH; complete treatment may even require alternatives to equation (1).

Even with these potential complexities, rates of ligand-exchange around dissolved complexes (Fig. 1a) and the rates of ligand-promoted dissolution (Fig. 3a) vary in a remarkably similar fashion. This similarity, along with the affects of different metals¹⁹, greatly simplifies the prediction of reactivity trends for surface reactions. For example, Ni(II) forms a strong complex with pyridine¹⁶, yet neither bipyridine nor terpyridine accelerate rates of water exchange at distal sites. The rate coefficient for H₂O exchange around Ni(H₂O)₆²⁺ (aq) is 0.32×10^5 s⁻¹ (refs 10–12), and the rate coefficients for H₂O exchange around the Ni(bipyridine)(H₂O)₄²⁺ (aq) and Ni(terpyridine)(H₂O)₃²⁺ (aq) complexes are 0.49×10^5 s⁻¹ (ref. 10) and 0.52×10^5 s⁻¹ (ref. 10). Pyridine ligands do not sufficiently weaken Ni(II)-oxygen bonds to enhance the reaction rates in the complex and will not appreciably affect rates of reaction at a surface either.

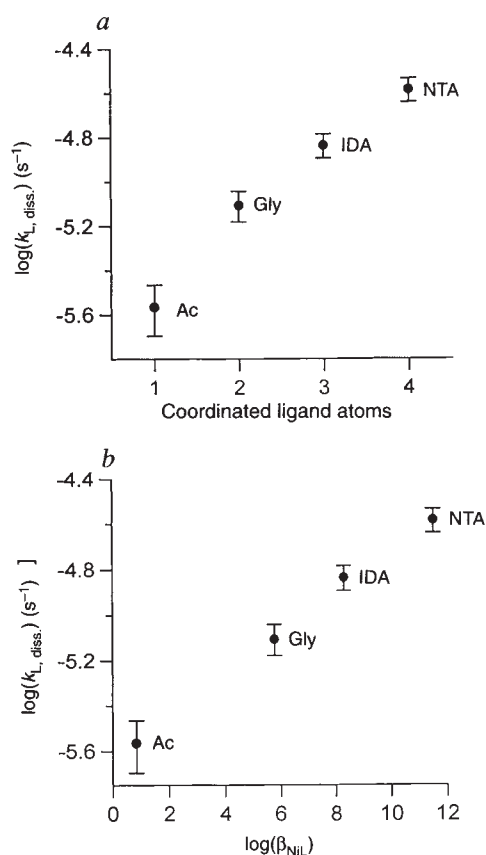


FIG. 3 a, Rate coefficients (k_L) for ligand-promoted dissolution of bunsenite (NiO(s)) at pH 6.0, as a function of the number of coordinated ligand atoms. The ligand structures are shown in Fig. 2. b, As a, but as a function of the metal-ligand stability constant (β_{NiL}) for the dissolved metal-ligand complex at equilibrium.

There are important environmental implications of this result. Equilibrium models of contaminant geochemistry are criticized²⁰ because rates of the important reactions are commonly slow and the rate coefficients unknown. We find that the rate coefficients of an important class of disequilibrium reactions are predictable, and that linear-free-energy correlations for wider ranges of complexes are possible^{19,21}. Furthermore, systematic study of a few ligands may serve to bound the reactivities of a wide range of organic compounds because steric crowding must ultimately limit the size, rigidity and number of ligands that can coordinate to a surface metal. Therefore, although much soil organic matter is of high molecular weight and uncertain structure, these polymers may act as a collection of smaller ligands with properties that can be estimated. □

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Evolution of East Pacific Rise hydrothermal vent fluids following a volcanic eruption

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STUDIES of sea-floor hydrothermal vent fluids have shown them to have stable characteristics on a decade timescale¹, with temperatures in the range $350 \pm 30^\circ\text{C}$ (ref. 2) and chemistries² that vary from vent to vent, which have most recently been interpreted to reflect phase separation within the hydrothermal system^{3–7}. Here we report measurements of vent fluid temperature and chemistry from $9^\circ 46.5' \text{N}$ on the East Pacific Rise, which show unprecedented variability on timescales of only a week. Our measured

temperatures range up to 403°C , placing the fluids unequivocally in the vapour field at the sampling conditions. Consistent with the fluids being in the vapour phase are the lowest chlorinities and silica contents, and highest hydrogen sulphide contents, yet reported for sea-floor vent fluids. These unusual fluid characteristics are the result of a volcanic eruption having occurred at this site, within weeks of when the first measurements were made⁸. The hydrothermal system in the immediate post-eruptive period is thus characterized by previously unknown temperature and chemical characteristics, necessitating revisions to models of hydrothermal fluxes to the oceans.

In April 1991 evidence of a very recent lava flow was discovered on the axis of the East Pacific Rise (EPR) between $9^\circ 45'$ and $9^\circ 52' \text{N}$ at $104^\circ 17' \text{W}$ ⁹, confirmed by radiometric dating of the lavas⁸ to have occurred no more than two weeks before our first dive at the sight in the deep submergence vehicle *Alvin*. The young hydrothermal system was characterized by voluminous, disorganized venting of hydrothermal fluids from the sea floor, and the virtual absence of sulphide- or sulphate-constructional features accompanying the high-temperature hydrothermal discharge. The 'A' vent area located at $9^\circ 46.5' \text{N}$ and $104^\circ 16.8' \text{W}$ consists of three discrete, high-temperature vents. The 'Aa' vent is located on a bench at a depth of 2,532 m on the western wall of the axial summit caldera (ASC), $\sim 3 \text{ m}$ below the top of the wall and 3 m above the bottom of the ASC which is a relatively narrow 60 m in this area¹⁰ (Fig. 1). The 'Ab' vent is located on the same bench, $\sim 2 \text{ m}$ east of the Aa vent, and closer to the eastern edge of the bench. The 'Ac' vent was first discovered in 1994 and is on top of the ASC wall, slightly north and above the Aa and Ab vents ($\sim 31 \text{ m}$ total distance). On 10 April 1991 the Aa vent consisted of smoky fluids issuing directly from a talus pile for a length of $\sim 1.5 \text{ m}$, with a measured temperature of 390°C . The temperature was confirmed using two independent temperature-measuring systems, the *Alvin* high-temperature probe and the NOAA manifold sampler temperature probe. On this date no sulphide- or sulphate-constructional features had formed. On 17 April 1991 this vent was resampled and a temperature of 396°C was measured. On 24 April 1991, Aa vent was resampled for the third and last time during the 1991 cruise and a temperature of 403°C was measured. Based on the equation of state for sea water at 250 bar (the sea-floor pressure), the two-phase curve for sea water is at 388°C (ref. 11), hence any measured temperatures greater than this value would put the fluids in the vapour field (Fig. 2). During the three 1991 samplings of this vent it was therefore in the vapour stability field, making this the first time that vapour-phase fluids have been unequivocally sampled, providing direct evidence that phase separation occurs at typical ridge-crest depths. As the temperature of the fluids increased over two weeks, so too did the hydrogen sulphide content, while the chlorinity and silica contents decreased (Table 1).

In March 1992 the Aa vent was resampled on two *Alvin* dives, two days apart. The temperature of the vent (332°C , measured on both dives) was significantly cooler than 11 months before, and the chemistry of the vent was also significantly different. There was a slight change in composition between the two 1992 samplings, suggesting the system had not reached steady state. By March 1992, Aa vent was characterized by an incipient sulphide mound with several $\sim 5\text{-m}$ -high sulphide spires actively venting fluid, and inactive fallen sulphide spires were already lying on top of the mound. The Ab vent, characterized by a venting sulphide chimney $\sim 5 \text{ m}$ high, was also sampled during the 1992 cruise, and had a distinctly different chemistry and temperature than the Aa vent located a few metres away.

In March 1994 both the Aa and Ab vents appeared much as they had in 1992, although the area around them now had a significant colony of serpulid worms. The temperature of the Aa vent was essentially unchanged from 1992, and the temperature and chemistries of the Aa and Ab vents were essentially identical when both were sampled on 12 March 1994. Aa was resampled

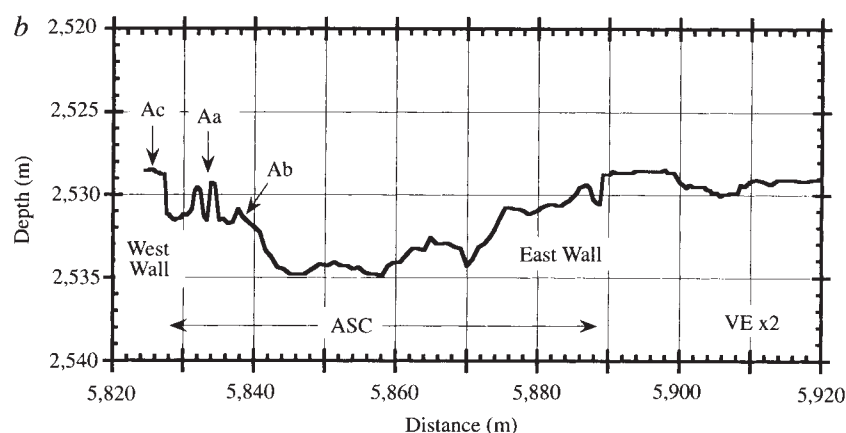
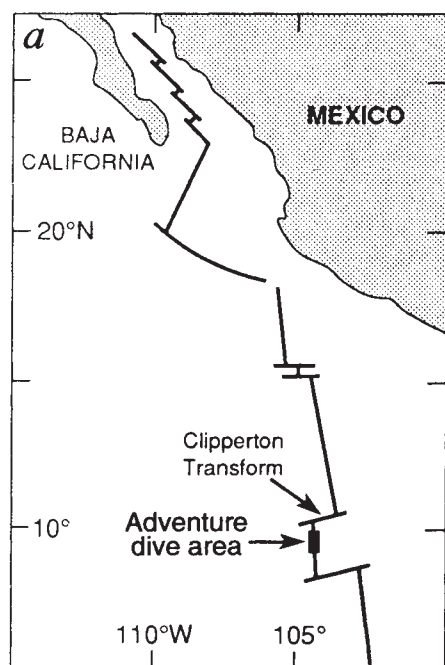


FIG. 1 *a*, Location of the Adventure (*Alvin* diving on the *Venture*) hydrothermal fields, which includes the A vent area study site. The location of the East Pacific Rise crest, including major transform faults, is designated by heavy lines. *b*, Cross-section of the axial summit caldera (ASC) in the A vent area, showing the relative locations of the three vents at this site. Note that vent Aa has multiple spires, the transect did not cross over the highest point of Ab vent, and Ac vent is located slightly north of the other vents. VE, vertical exaggeration.

on 26 March 1994, and Ab was resampled on 1 April 1994. These second 1994 samplings had distinctly different silica contents than the 12 March 1994 samplings, and these values have changed in concert. Short-term variability (on a timescale of days) in the silica content, also observed during the 1992 cruise, therefore continues into 1994. In searching for these two vents a new vent, Ac, was discovered during the 1994 cruise. Ac has significantly different chemistry from the other two vents, although its temperature is similar to Aa and Ab.

Thus even three years after the eruption, marked changes continue to occur on a timescale of weeks, the chlorinities remain below the sea water value, and the H_2S concentrations are high compared to other mid-ocean-ridge hydrothermal vents². As subcritical phase separation results in the formation of a vapour phase enriched in gases and depleted in salts with respect to the

starting fluid, the high H_2S and low chlorinities of the hydrothermal sea water are suggestive of continued phase separation at subcritical conditions. The measured conditions of 403 °C at 250 bar are just slightly below the critical point¹² of sea water, 407 °C and 298 bar. The extremely high H_2S contents in 1991 are the highest ever measured, and are lower limits to the actual concentrations because of some gas loss from the titanium water samplers. Compared to other vent fluids, a larger proportion ($\leq 40\%$) of the H_2S is from the reduction of sea water sulphate¹³. Although sea-floor hydrothermal activity has been previously viewed as a net sulphur sink¹⁴, the total amount of sulphur present in these vents in both 1991 and 1992 is greater than the sulphur content of sea water, suggesting that the net hydrothermal flux of this element may be positive, dependent on the duration and volume of the post-eruptive period.

TABLE 1 Changes in vent chemistry

Vent	Date	Temp. (°C)	H_2S^* (mmol kg ⁻¹)	Si (mmol kg ⁻¹)	Cl (mmol kg ⁻¹)
Aa.1	10 April 1991	390	$\geq 34 \pm 2^\dagger$	7.16 ± 0.04	80.3 ± 1.2
Aa.2	17 April 1991	396	$\geq 66 \pm 4$	3.72 ± 0.02	32.6 ± 1.6
Aa.3	24 April 1991	403	$\geq 71 \pm 10$ (110) [‡]	2.72 ± 0.02	45.2 ± 2.8
Aa.4	10 March 1992	332	30 ± 3	18.8 ± 0.1	286 ± 1
Aa.5	12 March 1992	332	32 ± 2	17.9 ± 0.1	261 ± 1
Aa.6	12 March 1994	334	10.7 ± 0.2	16.5 ± 0.1	398 ± 1
Aa.7	26 March 1994	324 (333) [§]	11.2 ± 0.1	17.5 ± 0.1	397 ± 1
Ab.1	10 March 1992	348	35 ± 5	13.4 ± 0.1	185 ± 5
Ab.2	12 March 1994	322	11.3 ± 0.3	16.6 ± 0.1	397 ± 1
Ab.3	1 April 1994	325	11.5 ± 0.1	17.5 ± 0.1	392 ± 2
Ac.1	12 March 1994	329	11.3 ± 0.1	18.6 ± 0.1	453 ± 1
Sea water		2	0	0.155 ± 0.005	540 ± 2

* Methods as in ref. 14, H_2S also by iodometric titration.

[†] All values are for $\text{Mg} = 0$ mmol kg⁻¹ extrapolation. Errors are the greater of analytical error, or least-squares fit to the data.

[‡] Extrapolation of more-dilute vent samples which experienced less degassing is 110 mmol kg⁻¹, rather than the 71 mmol kg⁻¹ predicted by a least-squares fit to all of the data.

[§] Although 324 °C was the temperature measured with the *Alvin* temperature probe on this date, a recording (Hobo) temperature probe deployed in the vent from 12 to 28 March 1994 recorded 333 ± 1 °C, suggesting no drop in temperature occurred, and that the lower temperature measured with the *Alvin* probe is due to incomplete insertion into the vent orifice.

^{||} Sea water contains ~ 28 mmol kg⁻¹ sulphur as SO_4 .

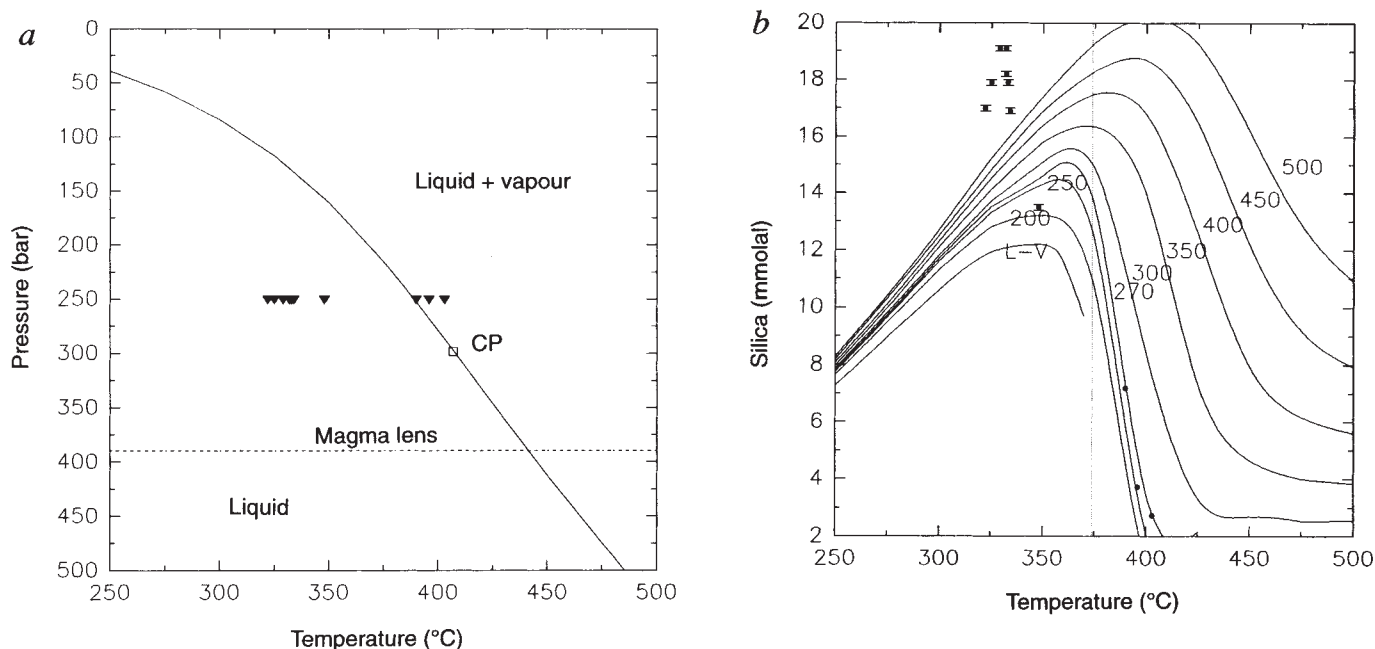


FIG. 2 a, Two-phase curve for sea water. Conditions at times of sampling are noted at sea-floor pressure of 250 bar. Note that several of the points are in the vapour field. CP, critical point of sea water. The dashed line labelled 'Magma lens' is superimposed on the figure, and indicates the depth at which the magma chamber is inferred to exist⁹, assuming hydrostatic pressure. b, Isobars of the solubility of silica in equilibrium with quartz in distilled water, calculated from equation in ref. 15 and the Haar equation. The pressure (in bars) is shown on, or to the right, of each isobar. L-V designates the liquid-vapour curve. Solubility is a function of salinity in this system; for the low-salinity 1991

samples, distilled water is more representative than sea water. (There is also experimental confirmation of quartz solubility in the vapour phase in distilled water²³.) Dotted line, critical point for pure water. Data from Table 1 are shown (black dots), 1 σ error bars are also shown or are smaller than the symbol. Samples taken in 1992 and 1994 are shown only for comparison, as their salt content requires different isobars to be drawn. (For this range of conditions, increased salt will increase the solubility, hence the pressures for the 1994 samples will be lower than they appear to be here.)

The silica contents of the 1991 samplings are the lowest observed to date from high-temperature sea-floor vents. Previous field and experimental results have shown that the silica contents reflect equilibration with quartz^{15,16}, which is found as an alteration mineral in these systems¹⁷. Earlier speculation was that the temperature of sea-floor vents would maximize at 350–375 °C, because the decreasing silica solubility at higher temperatures would cause fissures to rapidly seal, and hence the process would be self-limiting¹⁸. The observation of decreasing silica contents over two weeks in 1991 suggests that the low values are not simply an artefact of insufficient time to reach equilibrium, but instead reflect new conditions of equilibration as the temperature increases. The values observed in 1991 are consistent with equilibration with quartz at depths <200 m below the sea floor¹⁵; this is much shallower than in previous studies¹⁵. In 1991 the chloride contents were much less than the seawater value. To generate the observed chloride contents (Table 1) at the measured temperatures requires phase separation to have occurred at pressures of ≤ 270 bar, that is ≤ 200 m below the sea floor¹¹. The presence of a very shallow heat source, ≤ 200 m below the sea floor is unusual compared with the usual geophysically inferred depths for a fast-spreading-centre magma lens, and is much shallower than the inferred depth of 1.4 km for the magma chamber along this segment of ridge¹⁹. Harding *et al.*²⁰ have shown, based on seismic data, that the base of layer 2A, the sheeted-dyke to extrusive transition, is ~ 150 m deep in this area. The large increase in permeability at the sheeted-dyke/extrusive transitional²¹ may cause a pressure drop and induce phase separation, with its attendant volume increase, to occur at this depth. Three independent lines of evidence (silica geobarometry, chloride content as generated by the conditions of phase separation, and geophysical evidence on the depth of dyke intrusions and increased permeability) are all consistent with the heat source

and zone of water-rock reaction being a dyke intrusion within the upper 200 m of the oceanic crust. The observed fluid compositions are compatible with generation from sea water/basalt reaction, and do not require 'magmatic' inputs.

By 1992 and 1994 the temperatures were lower, and the chlorinities and silica contents significantly higher, than in 1991. As all the chlorinities remain below that of sea water, the fluids must have phase-separated, requiring them to have been at temperatures ≥ 388 °C within the oceanic crust. We have evidence from another vent at 9° 16.8' N on the EPR that the brine is stored within the oceanic crust and exits at a later time²². We cannot yet determine if the cooler measured temperatures are a result of conductive cooling or of mixing with cooler sea water. The higher silica contents in 1992 and 1994 require significantly greater depths of reaction within the oceanic crust, at least 500 m below the sea floor. The variations in silica between the repeat samplings during the 1992 and 1994 cruises are well outside the analytical error for these measurements. Although we do not completely understand the mechanism for these compositional changes occurring on the short timescales observed, it should be noted that for the subsurface conditions in this system changes as small as 10 °C in the temperature of reaction/equilibration between the water and the rock, or changes in pressure of <50 bar, could cause the observed changes. The changing silica concentrations are therefore indicative of continuing instabilities in the system at depth, the cooling of the dyke intrusion, and general deepening of the reaction zone. Temporal variability is therefore continuing for at least three years after the eruptive event, in marked contrast to the great stability on timescales of a decade in the chemical composition of vent fluids from areas in which no eruptive events have been observed¹. The unique chemical composition and continued temporal variability on the timescale of years after a volcanic eruption may have a profound

influence on the net chemical flux from sea-floor hydrothermal systems. Our present knowledge of the earliest stages of these hydrothermal systems, and their linkage to the size and frequency of volcanic events is at present too poor to evaluate quantitatively their net effect on heat and chemical fluxes. □

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Recent deformations of the deep continental root beneath southern Africa

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SEISMIC anisotropy, manifested by the splitting of shear waves into two orthogonal polarizations, provides evidence for deformation in the Earth's upper mantle. Where such deformation has been inferred beneath Precambrian cratons, it has been interpreted either as related to recent plate motion^{1,2}, or inherited from the Precambrian^{3,4}. Here we present data from a portable seismograph array in the Kaapvaal craton of South Africa, which indicate that the inferred direction of mantle flow underneath the array is close to the direction of absolute plate motion for southern Africa since the end of the Jurassic period. Such an alignment has also been reported for the North American craton², suggesting that the flow in both regions is related mainly to shearing of the sublithospheric mantle by the plate above. The old continental roots that are likely to exist in this depth range^{5,6} must therefore be deformed by the plate motion, but the deformations are not strong enough to be seen in the presently available seismic tomography data.

Shear-wave splitting in the mantle is related to the lattice preferred orientation of olivine, which depends on finite strain. Axes [100], [010] and [001] in olivine tend to become aligned with the longest, shortest and intermediate axes, respectively, of the strain ellipsoid^{7,8}. The most useful method for measuring shear-wave splitting in the mantle is based on the analysis of intermediate-period (3–15 s) recordings of SKS and similar seismic

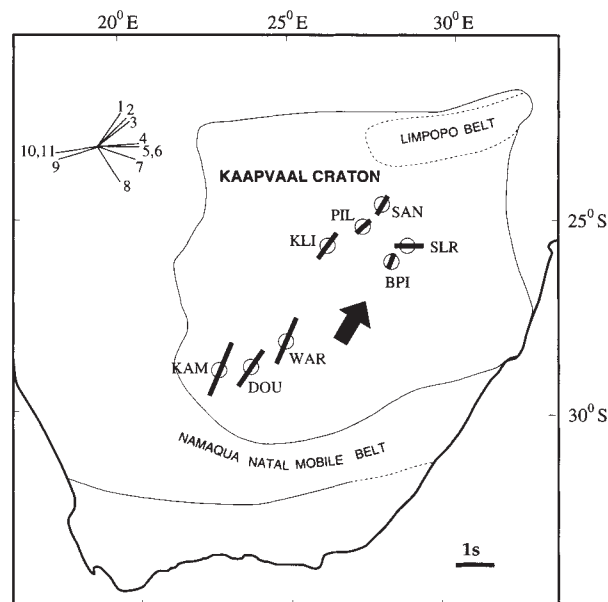


FIG. 1 Map of southern Africa. Boundaries of the Kaapvaal craton are adopted from ref. 20. The direction of polarization of the fast split wave at every station is shown by a straight line; the length of the line is proportional to the time lag δt of the slow wave (see scale bar). The data for station SLR are taken from ref. 2; all other data were obtained in this study. The direction of APM since the end of the Jurassic period is shown by an arrow. Back azimuths of the recorded events are shown in the upper part of the figure.

phases⁹. These phases propagate through the Earth's core and arrive at the receiver in a near-vertical direction. Ellipticity of the horizontal motion in these phases is controlled almost exclusively by azimuthal anisotropy underneath the receiver. So far, most of the data on shear-wave splitting in the subcratonic upper mantle have been obtained from North American stations, and they lead to controversial views on the origins of the inferred deformations. The observations within the Kaapvaal craton expand this data base significantly and help us to understand better the nature of the phenomenon. Seismic observations were carried out at seven digital broadband seismograph stations in a corridor 700 km long crossing the craton in a southwest–northeast direction (Table 1 and Fig. 1). This portable array was fully operational from the end of 1989 until the end of 1990. The seismographs used in the experiment are described elsewhere¹⁰.

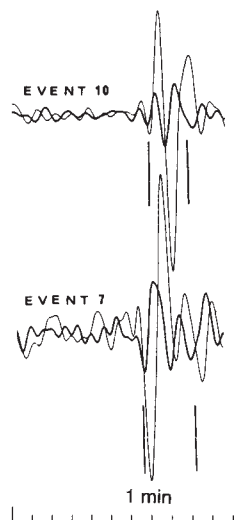
We invert the records of SKS and similar phases for polarization direction of the fast split wave (α ; measured clockwise from north) and time lag of the slow wave (δt) by using the algorithm given in ref. 1. Polarization of the fast wave is roughly parallel to the [100] axis in olivine. The optimum pair of α and δt is found by minimizing the r.m.s. difference (E) between the

TABLE 1 Seismograph locations and anisotropy parameters

Station	Latitude (deg.)	Longitude (deg.)	Events*	α (deg.)	δt (s)
BPI	−26.175	28.030	1,2,5,9,10	20	0.4
WAR	−28.377	24.893	4	20	1.3
DOU	−29.115	23.865	2,10,11	40	1.1
KAM	−29.133	23.125	2,6	20	1.4
SAN	−24.626	27.618	1,2,7,9,11	30	0.7
PIL	−25.221	27.101	1,2,3,7,8,9,10	50	0.6
KLI	−25.853	26.272	1,7,10	40	1.0

* The back azimuths of these events are shown in Fig. 1.

FIG. 2 Examples of the R - and T -component records of station PIL; the T component is shown as a bold line. The vertical lines indicate the time window used to calculate the penalty function E . Parameters of the events: number 7; date 30 December 1990, epicentral distance 117° , back azimuth 111° ; number 10; date 17 October 1990, epicentral distance 93° , back azimuth 256° .



observed transverse component (T) of SKS and the theoretical T component corresponding to the observed radial component (R). This pair is determined by a grid search over the values of the parameters between 0° and 180° and between 0 and 2 s with increments of 10° and 0.2 s for α and δt , respectively. A penalty function E is computed for every individual record and for the whole set of records of the seismograph station. Combined processing of all records gives the best final estimates of the parameters for the station concerned. These estimates are related to the nearest vicinity of the station within the first Fresnel zone. Seismic phases suitable for the above analysis were found in the records of 11 events. At most stations, the estimates of the parameters of anisotropy were obtained by processing the records of several events with differing back azimuths (Table 1). The records were individually filtered in the period range between 3 and 15 s to attain the highest possible signal-to-noise ratio.

Representative examples of the records, both for station PIL, are shown in Fig. 2. In the records of SKS and SKKS with strongly different back azimuths, a signal in both components with an amplitude much higher than the level of noise can clearly be seen. There is a quarter-period phase shift between the components, which is a diagnostic property of azimuthal anisotropy⁹. Positions of the minima of the respective penalty functions are reasonably close to each other (Fig. 3), and a similar solution is obtained for the whole set of records of PIL. The data for almost all other stations are of comparable quality: the scatter of individual estimates is within the range $\pm 15^\circ$ for α and ± 0.3 s for δt . Accuracy of the final estimates is generally $\pm 10^\circ$ for α and ± 0.2 s for δt . The data of SAN present an exception: for the back azimuths around 40° , 110° and 250° , the related estimates of α and δt are 140° , 90° and 40° and 2 s, 2 s and 0.5 s, respectively. This anomalous variability is probably a result of strong lateral heterogeneity of the Earth near SAN. This station is located close to the station PIL where the variations are in the normal range. Hence the anomalous structure should be shallow, perhaps crustal. In spite of scatter in the individual estimates, combined processing of all records of SAN gives a penalty function with well defined minimum. At all stations of our array, the resulting values of α are in the range of 20° – 50° (Table 1 and Fig. 1).

In Fig. 1 we also show, from refs 2 and 4, the data for station SLR and the Global Digital Seismograph Network. The value of α for SLR ($\sim 90^\circ$) is quite different from those for the other stations. This difference probably reflects complexity of the Earth's structure near the northeastern end of the corridor rather than an error of measurement. The change of the direction of

anisotropy from 90° at SLR to 20° at BPI within a distance of only 60 km could be explained by (for example) the presence of a shallow laterally variable anisotropic layer.

The average azimuth of polarization of the fast wave for all stations in Fig. 1, except SLR, is close to 30° . If this direction was related to ancient deformations, it would probably be aligned with the main structural boundaries: the Limpopo and Namaqua Natal belts (Fig. 1). This, however, is not the case. On the other hand, this direction practically coincides with the absolute plate-motion (APM) direction of southern Africa in the hotspot reference frame from the end of the Jurassic period until present¹¹. During this time, the plate shifted by $\sim 3,000$ km. Within the North American craton, the fast direction of anisotropy is close to the present-day APM direction². This APM direction is related to the opening of the North Atlantic and has not changed significantly since the end of the Cretaceous period, 65 Myr ago¹¹. The relationship between the fast direction of anisotropy and the APM direction in southern Africa is, clearly, very similar to that in North America and suggests the same origin of the large-scale component of mantle anisotropy in both regions: resistive drag at the bases of the moving plate. Then the deformation near the base of the plate is in the form of progressive simple shear which orients the [100] axis of olivine in the direction of plate motion¹².

The mobility of olivine depends on temperature, and significant lattice preferred orientation in a large volume of the mantle may arise only¹³ at temperatures higher than $\sim 1,000^\circ\text{C}$. Underneath the Kaapvaal craton, this temperature is reached at a depth of ~ 150 km, as shown by the mineral equilibrium studies¹⁴ of kimberlite inclusions. This temperature estimate is remarkably consistent with the geotherm calculated from heat

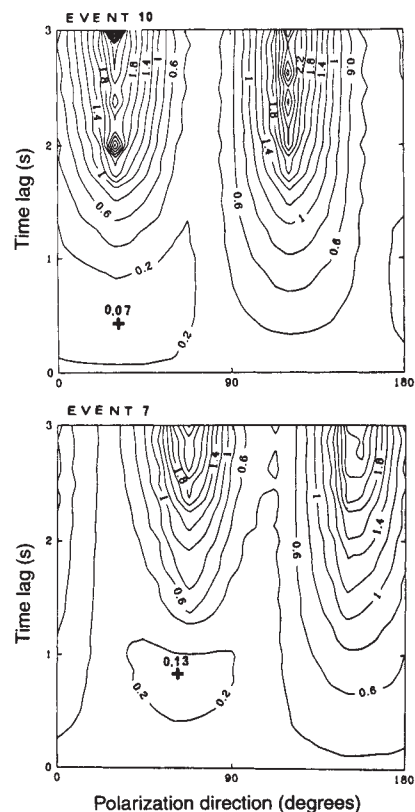


FIG. 3 Plots of the penalty function E (contours) for the records in Fig. 2. Position of the minimum value of E is marked by a cross and the value of E at that point. The optimum values of the polarization direction (α) and the time lag (δt) are 60° and 0.8 s for event 7, and 30° and 0.4 s for event 10.

flow data¹⁵. The present-day geotherm differs little from that in the Cretaceous period¹⁵. The properties of minerals at depths exceeding 400 km are unfavourable for the development of significant anisotropy¹⁶. Hence lattice preferred orientation created during the last 150 Myr in the mantle of the Kaapvaal craton is probably located at 150–400 km depth. To obtain δt of 1 s in a 250-km-thick layer, the required difference between the split wave velocities is 2%. This difference is ~ 3 times lower than the maximum values found in samples of upper-mantle rocks.

Seismic observations indicate consistently that the seismic velocities underneath Precambrian shields are anomalously high in the 150–400 km depth range⁵. Apparently, the old roots of the continents extend deep into the mantle and translate coherently with the plates⁶. The evidence of relatively young (younger than 200 Myr) deformations in the same depth range does not contradict the evidence of coherent motion. Significant lattice preferred orientation develops by intracrystalline slip when $\ln(c_1/c_2)$ or $\ln(c_2/c_3)$ —where c_1 , c_2 and c_3 are the largest, intermediate and smallest axes of the strain ellipsoid—are around 0.3 (ref. 8). This range of strains is reached when the root at a depth of 400 km is displaced laterally by 250 km with respect to a depth of 150 km. This shift (of the order of a few hundred kilometres at 400 km depth) could easily pass unnoticed in the presently available seismic tomography data. Although silicate inclusions in diamonds from the Kaapvaal craton yield Archaean model ages²¹, implying no significant displacement of the Archaean cratonic root, we do not consider this to be inconsistent with our model, as the diamonds come from depths of less than ~ 200 km, whereas the main part of the deformed zone is deeper than this.

Our conclusion does not mean that older deformations are absent in the subcratonic upper mantle. A strong contribution

from fossil anisotropy at shallow depth can be suspected at station SLR. At other stations, small-scale frozen anisotropy in the top of the upper mantle might be a reason for the lateral variations of the estimates of α and δt . Some effects of layered anisotropic structure in the seismic wavefield can be used to infer the parameters of anisotropy in the layers^{17–19}, but these effects are either too weak at most of our stations, or the amount of data is insufficient to identify them. $\square$

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Energy-saving gait mechanics with head-supported loads

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IN many areas of the world that lack a transportation infrastructure, people routinely carry extraordinary loads supported by their heads, for example the Sherpa of the Himalayas and the women of East Africa. It has previously been shown that African women from the Kikuyu and Luo tribes can carry loads substantially more cheaply than army recruits¹; however, the mechanism for their economy has remained unknown. Here we investigate, using a force platform, the mechanics of carrying head-supported loads by Kikuyu and Luo women. The weight-specific mechanical work, required to maintain the motion of the common centre of mass of the body and load, decreases with load in the African women, whereas it increases in control subjects. The decrease in work by the African women is a result of a greater conservation of mechanical energy resulting from an improved pendulum-like transfer of energy during each step, back and forth between gravitational potential energy and kinetic energy of the centre of mass.

Luo women, from the western flatlands of Kenya, carry loads on the top of their heads in an upright posture, whereas the Kikuyu women, from the rugged central highlands, carry loads supported by a strap that is looped across their foreheads, in a

forward-leaning posture, presumably so they can see where to place their feet. There are two intriguing aspects of the energetics of these women that have defied explanation^{2,3}: they can carry loads of up to 20% of their body weight with no demonstrable increase in oxygen consumption rate; and they can carry loads of up to 70% of their body weight, at all but the slowest walking speeds, much more economically than either trained people using backpacks, or untrained people using their heads or backpacks¹. Further study on West African women carrying head-supported loads has confirmed and extended these findings⁴.

In this study, two women carrying loads Kikuyu style, two women using the Luo style, and one woman able to use both styles, walked at 0.8–1.6 m s⁻¹ across a force platform mounted at ground level in the middle of a walkway. The gravitational potential energy and kinetic energy of the common centre of mass (body plus any load) were calculated from the measured forces⁵. For comparison, similar experiments were made on six male and six female Europeans carrying back-supported loads.

The walking gait is characterized by cyclic, out-of-phase, fluctuations in the height and forward velocity of the centre of mass of the body (as in a pendulum). This is because, during each step, the centre of mass is successively behind, or in front of, the point of contact of the foot on the ground. When the centre of mass is behind the point of contact, the link to the ground causes a forward deceleration (therefore a decrease in the kinetic energy of forward motion, $E_{cm,k}$) and a vertical rise in the centre of mass (therefore an increase in gravitational potential energy, $E_{cm,p}$); some of the $E_{cm,k}$ is converted into $E_{cm,p}$. As the centre of mass moves forward of the point of contact on the ground, the link to the ground allows a decrease in the height of the centre of mass and a concomitant increase in the forward velocity, as some of the $E_{cm,p}$ is converted back into $E_{cm,k}$. If the movement of the centre of mass were equal to that of an ideal, frictionless pendulum, the fluctuations of $E_{cm,k}$ and $E_{cm,p}$

would be equal and opposite, the total energy of the centre of mass ($E_{cm,tot}$) would be constant, and no external work (W_{ext}) would be required to maintain the motion. However, people are not ideal, frictionless pendulums, the kinetic and potential energy is not perfectly conserved, and consequently the $E_{cm,tot}$ fluctuates (Fig. 1). Nevertheless, the increments in $E_{cm,tot}$ (W_{ext}) are less than the sum of the increments in $E_{cm,p}$ (work against gravity, W_v) plus the increments in $E_{cm,k}$ (work to accelerate forward, W_f), indicating that at least some energy is conserved. The recovery of mechanical energy, resulting from this transfer between potential and kinetic energy, is $R = 100 (W_f + W_v - W_{ext}) / (W_f + W_v)$. In an ideal, frictionless pendulum, R would be 100%; in normal, unloaded walking at the optimal speed, R attains a maximum of 65% (ref. 6).

The energy curves of the unloaded African women are very similar to those of the unloaded control subjects (Fig. 1, left column). However, when carrying loads, the African women are more able to transfer their potential energy into kinetic energy, and vice versa, so the total energy curve is more constant, showing that less external positive work is done (Fig. 1, right column). The recovery of mechanical energy via this pendular transfer increases with increasing load in the African women (occasionally attaining values over 80%), whereas it remains about constant or increases slightly in the controls (Fig. 2, top). As a

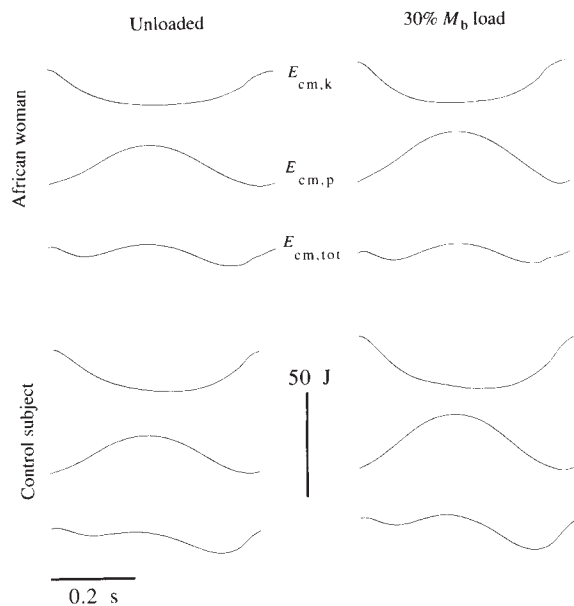


FIG. 1 Kinetic energy due to the forward velocity ($E_{cm,k}$, top curve in each panel), energy due to the vertical movements ($E_{cm,p}$, middle curve, mainly gravitational potential energy, but also including kinetic energy due to the vertical velocity), and total mechanical energy ($E_{cm,tot}$, lower curve) of the combined centre of mass (body plus any load) for a 63.8 kg African women (top panels) and a 60.7 kg control subject (bottom panels) walking without load (left panels) and while carrying a load equivalent to about 30% of their body weight, M_b (right panels). Note the similarity in the shape and amplitude of the traces for the unloaded subjects, indicating that there is little difference between the unloaded gaits of the African woman and the control. The load increases the amplitude of the $E_{cm,k}$ and $E_{cm,p}$ curves in both subjects but, because of the increased energy recovery in the African woman, her $E_{cm,tot}$ is flatter than that of the control, and her W_{ext} is relatively small as a result. The traces are original records for walking at a speed of 1.14 (upper left), 1.24 (upper right), 1.31 (lower left) and 1.33 (lower right) ms^{-1} ; each trace is within ± 1 s.d. of the average W_f , W_v , W_{ext} , and energy transfer in their respective experimental group. Traces were obtained by having the subjects walk across a $1.8 \text{ m} \times 0.4 \text{ m}$ force platform ($6.0 \text{ m} \times 0.4 \text{ m}$ in the case of the controls) sensitive to the force exerted in the forward and vertical directions⁷.

consequence, the weight-specific external work decreases with increasing load in the African women, whereas it increases in the controls (Fig. 2, bottom).

The increase in energy expenditure of African women and control subjects can be inferred from the increase in total mechanical work done (W_{tot}), assuming that efficiency is unaffected by load. The increase in W_{tot} , in turn, can be calculated, as described in the legend of Fig. 3, from the external mechanical work, assuming that the internal mechanical work (the work required to accelerate the body segments relative to the centre of mass) is unaffected by load. Because the step frequency, which is the same in the African women and the controls, is unaffected by load, it is reasonable to assume that the internal mechanical work is also unaffected by load.

That the African women can carry loads of up to nearly 20% of their body weight with no measurable increase in energy expenditure (oxygen consumption rate) is accounted for by the finding that, over the same load range, the W_{tot} does not increase (Fig. 3, filled circles). This is in contrast to the proportional increase in W_{tot} observed in the control subjects over the same load range (Fig. 3, open circles). Further, that the increment in energy expenditure of African women is much less than that

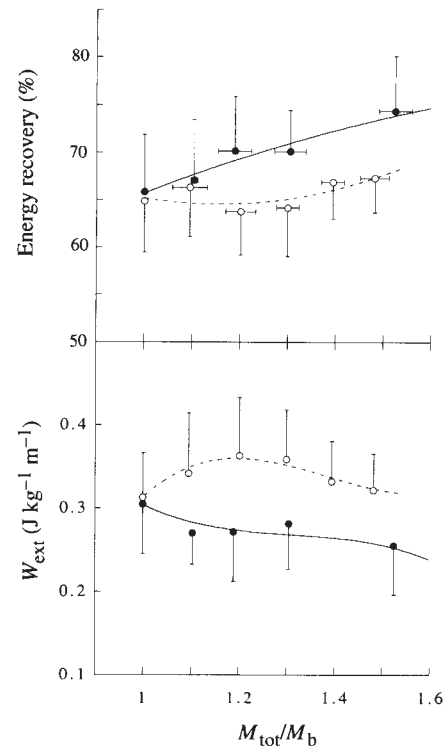


FIG. 2 Top, the energy recovered via the transfer between potential energy ($E_{cm,p}$) and kinetic energy ($E_{cm,k}$), about the same in all unloaded subjects, increases with increasing loads in the African women (filled circles), whereas it is nearly independent of load in the control subjects (open circles). On the abscissa, total weight (M_{tot}) is expressed as a multiple of body weight (M_b). Bottom, the weight-specific (body weight plus any load) external work done per unit distance decreases with loads in the African women (filled circles), whereas it increases with loads in the controls (open circles). The circles and bars represent the mean and standard deviation of the mean of the groups of trials based upon the following load ranges (loads are expressed as per cent of body weight; the numbers in parentheses represent the number of trials averaged for the African women and controls, respectively): 0% (121, 161); >0% to <15% (59, 85); 15% to <25% (73, 78); 25% to <35% (64, 73); 35% to <45% (0, 68); and 45% to 60% (25, 36). Fitting of the lines to the ungrouped data points of the African women (solid lines) or controls (broken lines) used either a second-order (top) or third-order (bottom) polynomial curve fit.

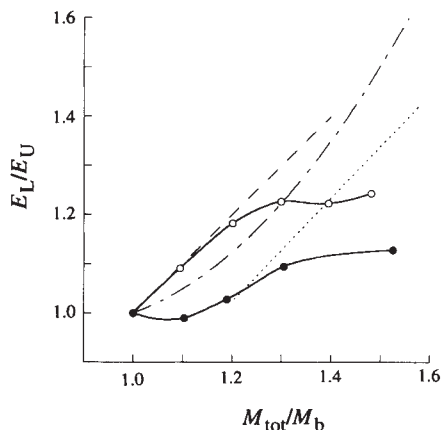


FIG. 3 The mechanical energy output and the total energy consumed when carrying loads is compared with the energy output or consumed when walking unloaded, as a function of load. The ratio of the total work done when walking with a load to the total work done when walking unloaded is shown for the African women (filled circles) and the control subjects (open circles). The dotted line is the straight line used by Maloiy *et al.*¹ to fit their Fig. 3 data of the ratio of oxygen consumption rate in African women carrying loads to their oxygen consumption rate when unloaded. Similarly, the dashed line, also from Maloiy *et al.*, is the relationship found for untrained adults carrying loads on their backs or their heads. The dot-dash line is the same ratio, calculated from Pandolf *et al.*⁸, for army recruits carrying backpacks. Note that the African women can carry loads of nearly 20% of their body weight with no increase in their total work, whereas, for the same loads, the control subjects' work increases in proportion to the load. For loads greater than 30% of body weight, both the African women and the controls show little further increase in the total work, indicating that the work ratio is no longer indicative of the oxygen consumption ratio, which increases continuously with load (dotted and dashed lines). However, the work ratio of the African women at these high loads is only about half that of the controls. The work ratio on the ordinate was calculated as:

$$\begin{aligned} \frac{W_{\text{tot,L}}}{W_{\text{tot,U}}} &= \frac{W_{\text{ext,L}} + 0.537W_{\text{tot,U}}}{W_{\text{tot,U}}} \\ &= \frac{\frac{W_{\text{ext,L}}}{W_{\text{ext,U}}} \cdot 0.463W_{\text{tot,U}} + 0.537W_{\text{tot,U}}}{W_{\text{tot,U}}} \\ &= \frac{W_{\text{ext,L}}}{W_{\text{ext,U}}} \cdot 0.463 + 0.537 \end{aligned}$$

on the assumption that the internal work, namely the work required to move the body segments relative to the combined centre of mass, is equal to the unloaded walking value (that is, $0.537W_{\text{tot,U}}$) (ref. 9); the U and L subscripts indicate unloaded and loaded measurements, respectively. The ratio $W_{\text{ext,L}}/W_{\text{ext,U}}$ was calculated for each load by multiplying the data in the bottom of Fig. 2 times the total weight (body weight plus any load).

of the control subjects (Fig. 3, dotted against dashed lines) is accounted for by the finding that the increment in W_{tot} by the African women is much less than that done by the controls at all loads.

The dissociation between the increase in energy expenditure and the increase in mechanical work for loads greater than 30% of body weight may be due to factors not measured here as mechanical work, but which nevertheless increase the energy expenditure at these high loads. These factors may include a decrease in muscular efficiency, an increase in isometric contractions required to maintain posture and support the load, and increase in the internal work due to movements of the load relative to the centre of mass. Alternatively, the skill of these women in carrying head-supported loads may require muscular

strength appropriate for the load. At very high loads in the African women, and at all loads in the control subjects, the required strength may not be available; this may also help explain the finding that obese African women do not exhibit the same economy of head-supported load carriage⁴.

In conclusion, African women can carry head-supported loads of up to 20% of their body weight for 'free', because their total mechanical work of walking does not increase. Furthermore, they carry loads of up to 70% of their body weight for a lower metabolic cost than control subjects carrying either head- or back-supported loads because they do less mechanical work. They conserve more mechanical energy through a more complete energy transfer, back and forth between the kinetic energy of forward motion and the potential energy of their centre of mass; in effect, they become better pendulums. □

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Use of implicit motor imagery for visual shape discrimination as revealed by PET

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POSITRON emission tomography (PET) can be used to map brain regions that are active when a visual object (for example, a hand) is discriminated from its mirror form. Chronometric studies^{1–3} suggest that viewers 'solve' this visual shape task by mentally modelling it as a reaching task, implicitly moving their left hand into the orientation of any left-hand stimulus (and conversely for a right-hand stimulus). Here we describe an experiment in which visual and somatic processing are dissociated by presenting right hands to the left visual field and vice versa. Frontal (motor), parietal (somatosensory) and cerebellar (sensorimotor) regions similar to those activated by actual^{4,5} and imagined^{6–8} movement are strongly activated, whereas primary somatosensory and motor cortices are not. We conclude that mental imagery is realized at intermediate-to-high order, modality-specific cortical systems, but does not require primary cortex and is not constrained to the perceptual systems of the presented stimuli.

Virtually all brain regions known to participate in the planning and execution of bodily movements (with the exception of primary sensorimotor cortex, see later) were activated

TABLE 1 Activation foci

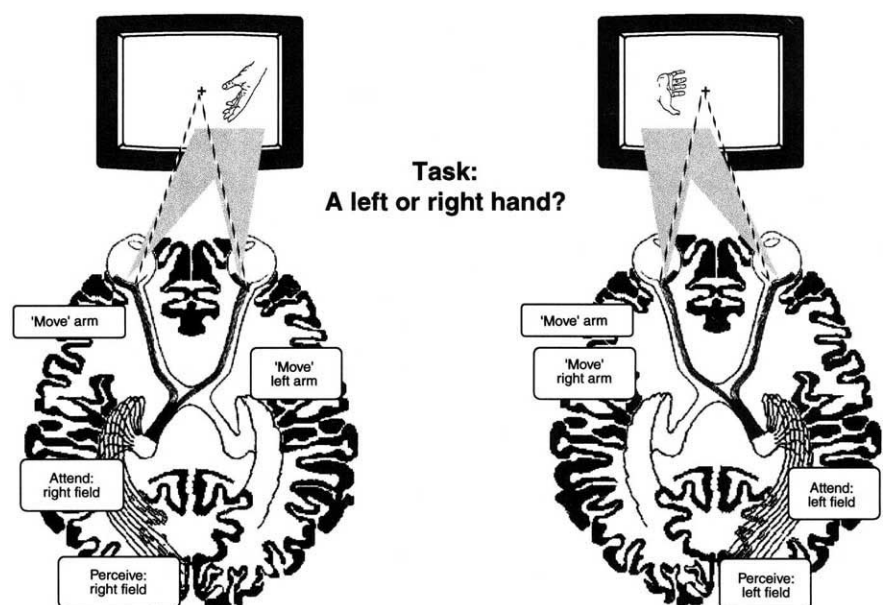
Plot	Area	Extent (mm ³)	Intensity (c.b.f. change)	Z-score	P	x mm	y mm	z mm	Plot	Area	Extent (mm ³)	Intensity (c.b.f. change)	Z-score	P	x mm	y mm	z mm
Left hands, right visual field									Right hands, left visual field								
3	BA6	504	5	3.1	**	-6	1	60	3	BA6	72	5	2.7	**	-4	-2	59
9R	BA40	584	6	3.5	****	38	-38	50	4R	BA6	160	5	2.9	**	32	-10	54
8L	BA7	752	5	2.8	**	-30	-60	50	3	BA6	168	5	2.6	**	-5	8	54
4R	BA6	544	5	2.8	**	26	-2	48	4L	BA6	568	6	3.3	***	-26	-10	54
4L	BA6	840	7	3.7	*****	-26	-12	48	4R	BA6	160	5	2.8	**	26	-2	54
8R	BA7	688	5	2.8	**	34	-54	48	8L	BA7	584	6	3.4	****	-30	-58	52
8R	BA7	600	5	2.7	**	28	-64	46	8R	BA7	152	5	3.1	**	22	-48	48
8L	BA7	536	5	2.9	**	-20	-64	46	8L	BA7	160	5	2.9	**	-19	-62	46
7L	BA6	784	7	3.9	*****	-2	16	46	4L	BA6	456	6	3.6	***	-38	-6	46
4L	BA6	648	5	3.1	**	-40	-8	46	8R	BA7	344	6	3.3	***	26	-56	46
8L	BA7/40/19	416	5	2.8	**	-34	-66	42	7L	BA6	208	6	3.5	****	-4	16	44
9L	BA40	80	5	2.6	**	-32	-58	40	8R	BA7	128	5	2.9	**	16	-66	42
9R	BA40	536	5	2.6	**	38	-56	38	9L	BA40	536	6	3.5	****	-42	-40	42
9L	BA40	992	6	3.5	****	-36	-42	38	9L	BA40	536	6	3.2	***	-34	-40	42
5R	BA44	656	6	3.4	***	42	4	34	9R	BA40	272	5	3.0	**	42	-30	40
5L	BA44	672	5	2.9	**	-44	0	32	8R	BA7	400	7	3.8	*****	27	-38	36
2	BA46/9	400	5	2.8	**	36	32	26	5L	BA44/6	96	5	2.7	**	-46	-1	32
6R	INS	496	5	3.1	**	27	20	6	2	BA46/9	80	5	2.8	**	-45	36	30
13L	CBM	368	6	3.4	***	-6	-44	-6	5L	BA44	88	5	2.7	**	-60	14	16
19R	CBM	632	5	2.9	**	38	-73	-16	6L	INS	32	5	2.6	**	-32	12	14
19L	CBM	872	6	3.4	***	-8	-76	-16	5R	BA44/45	112	6	3.4	****	56	24	12
13R	CBM	760	7	4.0	*****	46	-65	-18	19R	CBM	136	5	2.9	**	14	-67	-8
13L	CBM	512	6	3.1	**	-38	-52	-18	13L	CBM	104	5	2.8	**	-8	-44	-9
13L	CBM	360	5	2.8	**	-10	-36	-20	13R	CBM	112	5	3.0	**	20	-51	-14
19L	CBM	216	5	2.7	***	-51	-76	-24	13L	CBM	176	6	3.2	***	-11	-46	-14
13R	CBM	328	5	2.8	**	17	-48	-28	19R	CBM	272	5	3.1	**	42	-72	-22
13R	CBM	352	5	2.6	**	40	-48	-30	19L	CBM	80	5	3.0	**	-52	-76	-24
13R	CBM	392	5	2.8	**	32	-38	-32	19R	CBM	64	5	2.7	**	10	-70	-24
13R	CBM	352	5	2.6	**	24	-62	-36	19R	CBM	24	5	2.6	**	2	-80	-26
									19R	CBM	224	6	3.4	****	30	-68	-28
									13R	CBM	40	5	2.6	**	18	-48	-28
									19L	CBM	32	5	2.7	**	-10	-79	-32

Focal increases in brain blood flow in somatic sensory and motor areas occurring during each experimental condition (relative to rest) are listed. (A comprehensive listing of all brain areas active in these tasks is available in the BrainMap database or as Supplementary Information from *Nature*.) 'Plot' identifies the regional activation into which the focus was clustered (Fig. 2). 'Area' indicates the anatomic structure(s) activated by Brodmann area (BA) or structure name (INS, insula; CBM, cerebellum). 'Extent' is the total volume of all contiguous pixels above statistical threshold ($P < 0.005$) of each focus. 'Intensity' is the peak change in cerebral blood flow (c.b.f.; expressed in millilitres per 100 g per min) occurring within the activated site. 'Z-score' is intensity divided by image noise. 'P' is statistical significance: *, 0.005; **, 0.001; ***, 0.0005; ****, 0.0001; *****, 0.00005 (ref. 29). 'Coordinates' are expressed in mm from the anterior commissure: x, right(+)/left(-); y, anterior(+)/posterior(-); z, superior(+)/inferior(-). Coordinate and area follow ref. 30.

FIG. 1 Experimental concept. We hypothesized that left-right judgements of visually presented hands would elicit an implicit movement of the hand corresponding to the stimulus. Hemifield-based processes were dissociated from hemisoma-based processes using the principle of cerebral contralaterality. Left hands were presented predominantly to the right visual field; right hands were presented predominantly to the left visual field. Attention to one hemifield (for example, 'attend right field') and processing visual information from that hemifield (for example, 'see right field') were expected to activate the contralateral parietal and occipital areas. Implicit movement was expected to activate the dominant (left) frontal lobe, for higher-level, less specified planning (for example, 'move' arm) and in the hemisphere contralateral to the limb 'moved' for lower-level, more specified planning (for example, 'move' left arm).

METHODS. Subjects made a covert decision as to whether a visual stimulus was a left or right hand. Subjects fixated on a black dot in the centre of a video monitor as stimuli appeared singly either in the left or right visual hemifield. They were unable to see their own hands and were not allowed to make actual movements.

Physically adopting the posture of the presented hand in many instances would require coordinated activity at more than one arm joint, motion near extremes of joint limits, uncomfortable kinaesthetic sensations, and long trajectories³. There were two experimental tasks: a left-visual-field task and a right-visual-field task. In the left-field task, 80% of the stimuli were right hands, 20% were left hands. In the right field task, the ratios were reversed. Stimuli were presented at a mean



eccentricity of 3.2°, with the edges of the stimulus 0.6° (inner edge) and 5.6° (outer edge) from fixation. Stimuli were presented for 150 ms. The time between the offset of one stimulus and onset of the next was adjusted to allow just barely enough time for task performance, based on prior chronometric pilots. These conditions, allowing no time for introspection, heightened the implicit nature of the performance. The rest condition was fixation on the central dot.

by this visual shape task (Figs 1 and 2; Table 1). Prefrontal and insular premotor areas (clusters 2, 6), involved in global spatial and temporal schemata for movement preparation^{4,9}, were purely contralateral to stimulus handedness (somatic laterality). Supplementary motor area (SMA, cluster 3), active during imagined movement^{6,7} and important in motor memory and sequencing^{9,10}, was strongly activated in left brain for both conditions, reflecting cerebral dominance for motor programming (dominance laterality). Anterior cingulate, in a region probably projecting onto SMA¹⁰, showed dominance laterality. Superior premotor cortex (Brodmann area (BA) 6, cluster 4) activated bilaterally but more strongly in the left hemisphere, a motor-dominance laterality. Inferior premotor cortex (BA44/45, cluster 5) activated bilaterally with somatic laterality. Superior and inferior premotor areas are important for planning, initiation, guidance and execution of simple and skilled motor tasks and limb movements in extrapersonal space^{11–13}. Cerebellum and basal ganglia (clusters 13, 19), known to be active during actual movement^{5,11}, were bilaterally active. No activation was detected in primary somatic sensory or primary motor cortex, consistent with the absence of body movement during the task.

Posterior and inferior parietal cortex—multimodal association areas important to body schema, egocentric space and action

planning^{14–16}—activated bilaterally but showed a mix of visual, somatic and dominance lateralizations. For both conditions, superior parietal regions (BA7) were active bilaterally, with somewhat more activation in the hemisphere contralateral to stimulus hemifield (cluster 8, Fig. 2). We interpret this as a posterior attentional system aiding covert shifts of attention to the contralateral hemifield¹⁷. Inferior parietal (BA40) was bilaterally active with a hand effect stronger for right hands than for left (cluster 9, Fig. 2).

These somatic-motor activations support the principle that mental imagery is realized using modality-specific neural systems. This is most frequently hypothesized in the perceptual domain¹⁸. Namely, visual systems subserve visual imagery; auditory systems subserve auditory imagery. Our motor-system activations reflect implicit motor imagery (as argued below). These data confirm and considerably extend preliminary indications of correspondence between the neural systems for actual and imagined movement^{6–8}. Note that motor imagery was realized through neural systems of movement but did not require the primary sensory or motor cortices, perhaps unlike visual imagery¹⁸. In addition, these data confirm the hypothesis^{1–3} that motor imagery can be employed to solve problems posed in perceptual domains, using mental transformations of the viewer rather than of the viewed object. This supports the principle

FIG. 2 Activation cluster plot. Each circle designates a cluster of focal, task-induced increases in cerebral blood flow ($P < 0.005$). Circles depict the mean location and collective extent of one or more discrete foci (Table 1). Brain contours are coronal sections positioned +36 mm, +16 mm, -48 mm, and -60 mm from the anterior commissure. Probable Brodmann cytoarchitectonic areas are given parenthetically.

METHODS. Discrete activation foci (Table 1) were grouped into regional-activation clusters based upon probable Brodmann area and gyrus (Table 1). Clusters are represented as circles. The circle representing each cluster is centred on the mean location for all local maxima included in the cluster. The area of each plotted circle is an accurate 2D projection of the sphere—true to the scale of the section—whose volume equals the sum of the activated voxels of the clustered foci. Each voxel had a volume of 8 mm^3 . Clusters are plotted on the nearest coronal section.

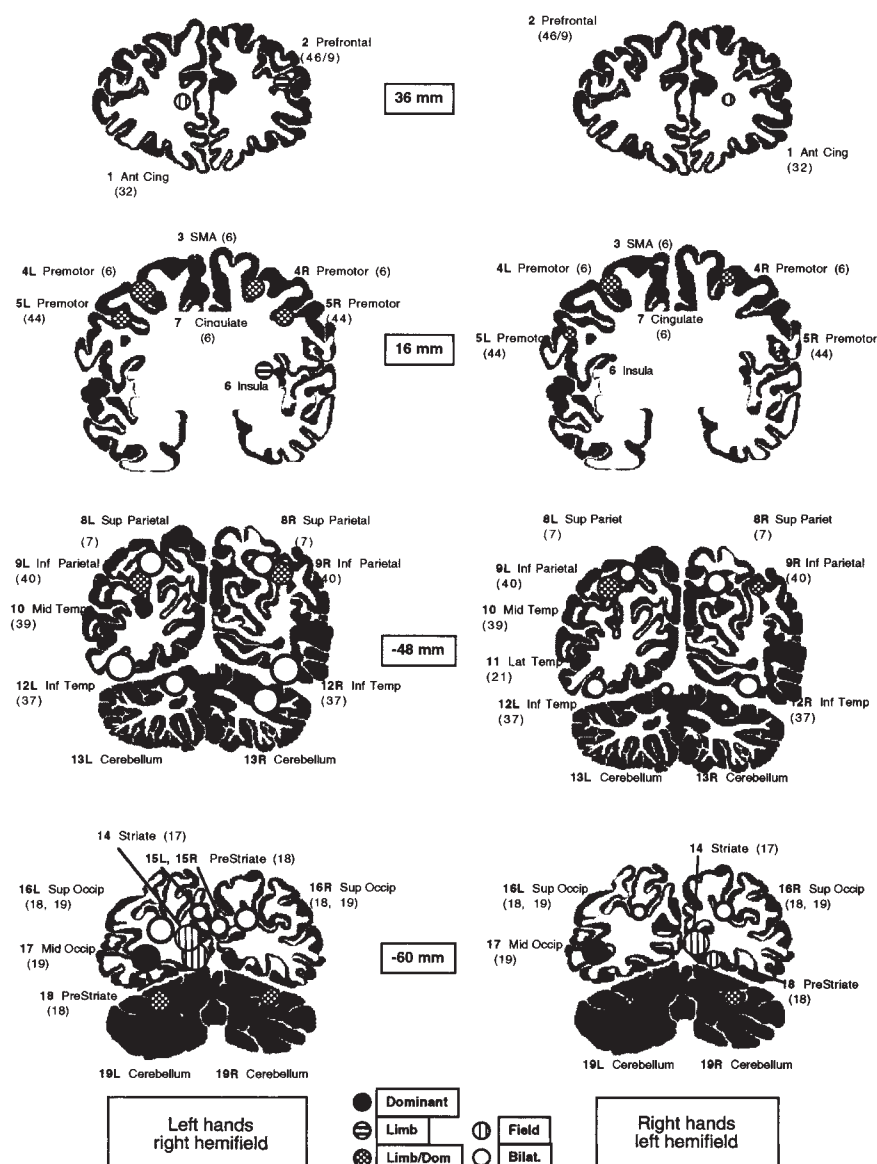
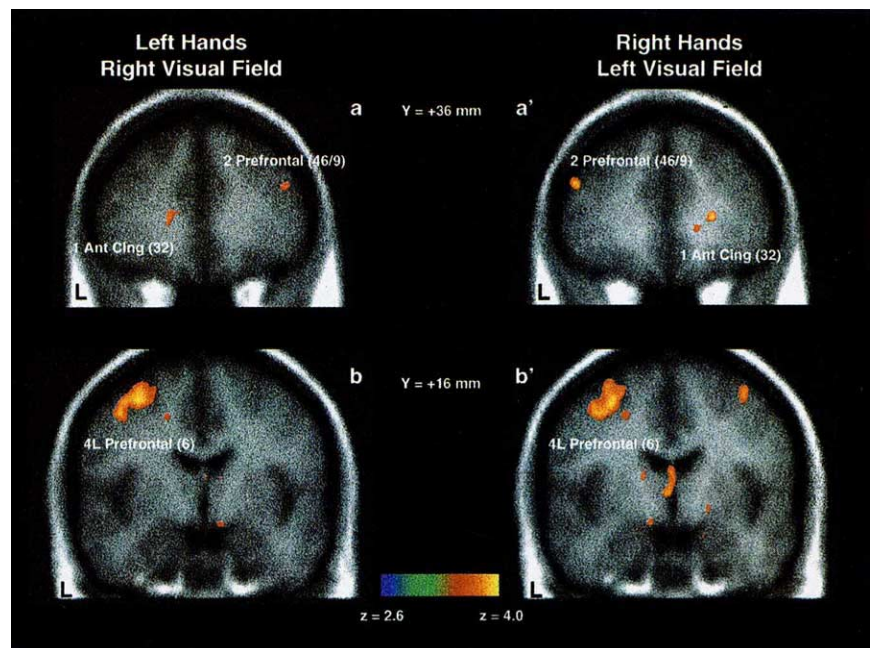


FIG. 3 PET/MRI superposition. Three types of hemispheric lateralization are illustrated: field effect, hand effect and dominance effect. The anterior cingulate (Ant Cing) remains contralateral to the visual field of the stimulus, a field effect (a, a'). Prefrontal cortex remains contralateral to the body-side requiring implicit movement, a hand effect (a, a'). Premotor cortex is stronger in the motorically dominant hemisphere (left hemisphere in our right-handed population), regardless of the visual field or body side of the stimulus, a dominance effect (b, b'). Images were formed by superposition of the averaged (grand mean) PET functional data (colour) onto the averaged (grand mean) MRI anatomical data (grey scale). PET data are z-scores (Table 1) displayed on a colour scale ranging from 2.6 (blue; $P < 0.005$) to 4.0 (yellow; $P < 0.00003$). Images are coronal sections positioned +36 mm (a and a'), and +16 mm (b and b') in front of the anterior commissure. Probable Brodmann cytoarchitectonic areas are given in parentheses.

METHODS. The subjects were seven, healthy right-handed normal volunteers (six men and one woman, ranging in age from 20 to 35 yr, with a mean of 24 yr). After giving informed consent, each subject underwent nine PET blood-flow scans, three in each experimental task and three in fixation-point rest. Images were averaged within condition for each subject. Images were spatially normalized into the bicommissural coordinate space²⁹ and then averaged within condition among subjects. The grand-mean image for each task was compared to control, forming grand-mean images of the task-induced changes in brain activity. Grand-mean images were converted to z-score images using the population variance²⁹. To ensure that each subject performed the task as requested, an experiment was performed at the conclusion



of the scanning session. In the post task, a hand was presented in central fovea until the subject pressed a left or right button to indicate whether it was a left or right hand¹⁻³. Subjects were fast and accurate overall and faster for the stimuli from the experiment than for other stimuli, thus exhibiting general and stimulus-specific practice effects of their implicit performance in the experiment.

that abstract reasoning relies on physical analogies, employing mental models of physical objects and actions^{12,19}.

Visual processing areas were highly active. Primary (BA17, cluster 14) and early extraprimary (BA18, cluster 18) responses were purely contralateral to visual field (visual laterality). Some superior occipital responses (BA18/19, cluster 16) showed weak visual laterality, or were bilateral (BA18, cluster 15). Inferior temporal cortex (BA37, cluster 12), involved in representing object-based information^{20,21}, activated bilaterally, reflecting its bilateral receptive fields²². Lateral, inferior occipital cortex (BA19, cluster 17) activated solely on the left in both conditions. This strongly supports prior observations indicating a specific role in assembling visual percepts and images for this left-sided region²³. Two regions in anterior cingulate, one rostral (BA32, cluster 1) and one inferior (BA24, 25; LH15, RH50, Table 1) also showed visual laterality. These probably reflect covert shifts of attention to the hemifield of the stimulus¹⁷.

Visual recognition of objects is an active process whereby visual percepts are mapped onto stored mental representations²⁴. When viewing objects at unfamiliar orientations, the recognition process is sometimes enabled by mentally reorienting the object^{25,26}. For example, identity judgments of pairs of similarly shaped but dissimilarly oriented objects require the imagined rotation of one object to the orientation of the other²⁷. That the time required for such operations is an approximately linear function of rotation angle is a finding now considered classical in cognitive science. Such results demonstrate that imagined spatial transformations of abstract shapes often are performed in a viewer-based or scene-based visual space²⁸. Our results and the chronometric studies¹⁻³ upon which our study was predicated demonstrate that this is not always the case.

Response times for handedness judgements of visually presented body parts are far better modelled in a somatic space—a biomechanical joint space—than in a visual space. Specifically, chronometry suggests that a somatic representation is moved from the observer's current body position to the stimulus' orien-

tation without violating joint constraints¹⁻³. In fact, the time required for this visual discrimination is proportional to that of actual movement³. We interpret this as the execution of an implicit movement, as actual movements do not occur, imagined movements are not requested, and subjects often are unaware of an imagined movement (Fig. 1 legend).

The experimental strategy used here is noteworthy. Typically, PET studies strive for reductionism, contrasting conditions that (putatively) differ by a single cognitive operation. Here we distinguished somatic from visual operations by exploiting known differences in lateralization (retinotopic versus somatotopic), rather than by a reductionist design. Although such paradigms have been used for chronometric studies of split-brain patients, this is their first adaptation to functional neuroimaging. As our two tasks were complementary, rather than reductionist, each was contrasted with (subtracted from) a neutral control state (Figs 1 and 3 legends). Comparison to a neutral baseline allows all brain areas participating in a task to be localized and lateralized, protecting against the effects of incorrect *a priori* judgements about the number and nature of cognitive operations (Donders' fallacy)²³. Such caution is advisable when exploring new paradigms and mental operations.

Choice of an experimental design based on differences in lateralization assumed that subjects would implicitly move only the correct hand. That is, the mental representation of their right hand is moved when presented with a right hand and vice versa. Prior chronometric studies¹⁻³ indicate that pre-attentive recognition of the presented hand's laterality is followed by a confirmatory implicit movement of the correct hand, which allows an explicit judgement of the handedness of the stimulus.

An unexpected, unprecedented but very robust finding was that right-field stimuli induced much more extensive (178%, on average) activations than did left-field stimuli. This was true for responses in both hemispheres. However, the intensity and number of foci in corresponding regions did not differ. This was true for all active regions except primary visual cortex (BA17),

which argues that the effect was not an artefact of apparatus or method. This observation implies that processing of information from the dominant (right) visual field is more extensive than that of information from the non-dominant (left) visual field, irrespective of the hemisphere or region within which this processing is realized. □

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Homeotic genes and the regulation and evolution of insect wing number

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THE evolution of wings catalysed the radiation of insects which make up some 75 per cent of known animals. Fossil evidence suggests that wings evolved from a segment of the leg¹ and that early pterygotes bore wings on all thoracic and abdominal segments². The pterygote body plan subsequently diverged producing orders bearing three, two or just one pair of thoracic wings. We have investigated the role of homeotic genes in pterygote evolution by examining their function in *Drosophila* wing development and their

expression in a primitive apterygote. Wing formation is not promoted by any homeotic gene, but is repressed in different segments by different homeotic genes. We suggest here that wings first arose without any homeotic gene involvement in an ancestor with a homeotic 'groundplan' similar to modern winged insects and that wing formation subsequently fell under the negative control of individual homeotic genes at different stages of pterygote evolution.

The *Drosophila* wing and haltere primordia can first be visualized as discrete clusters of *vestigial*-expressing (Fig. 1a) and *snail*-expressing³ (Fig. 1b) cells in the second and third thoracic segments of the stage 12 embryo. Antibodies specific for the *vestigial* and *snail* proteins serve as interchangeable markers for the flight appendage primordia because they label the same cells at different embryonic stages (Fig. 1c–h). Both genes have a role in formation of the flight appendages because *vestigial* mutants lack wings and halteres⁴ and weak *snail* mutants that survive to adulthood exhibit a hemithorax or missing haltere phenotype⁵.

The restriction of the flight appendage primordia to the two posterior thoracic segments of the *Drosophila* embryo suggests a role for homeotic genes in specifying which segments will bear wings. Because the *Antennapedia* (*Antp*) gene is expressed in the second and third thoracic segments during embryogenesis^{6,7}, this gene might regulate the formation of the wing and haltere primordia. Surprisingly, there is no requirement for *Antp* gene function during the formation of these primordia (Fig. 1i). This suggests that wing primordium formation as it occurs in the mesothoracic (T2) segment represents a 'ground state' of embryonic development without the input of the homeotic genes. Furthermore, this indicates that ectopic wing primordia found in other homeotic mutants (see below) result from derepression of the wing developmental programme, rather than the derepression of *Antp*.

It appears that, rather than *Antp* positively regulating wing and haltere formation in T2 and T3, other homeotic genes repress the wing primordia in different body segments. For example, the *Sex combs reduced* (*Scr*) gene is expressed in the labial and prothoracic (T1) segment^{8,9}. In *Scr* mutant embryos ectopic flight appendage primordia arise in the T1 segment, demonstrating that *Scr* normally represses wing formation there (Fig. 1j). Similarly, in mutants for the *Ultrabithorax* (*Ubx*) gene, which normally functions in both the metathoracic (T3) and first abdominal segment (A1)¹⁰, the haltere primordia in T3 expands to the size of the wing primordia (data not shown) and an ectopic primordium forms in the ventral region of A1 (Fig. 1k). The potential for wing formation exists in most or all trunk segments as illustrated by embryos lacking the *abd-A* gene (Fig. 1l) or the *Ubx* and *abdominal-A* (*abd-A*) genes (Fig. 1m) (see also ref. 11).

The experiments above address only the formation of the embryonic wing primordia and not the growth and development of adult wings from these primordia. Cells may respond differently to the expression of a particular homeotic gene at different stages of development¹²; thus *Antp* could function during later stages of wing formation. There are no genetic requirements for *Antp*, *Scr* and *Ubx* in the formation of adult wings (*Antp* is required in the mesonotum)¹³. Furthermore, we find that *Antp* protein expression is largely absent from the region of the growing third instar imaginal disc that will give rise to the wing itself (Fig. 2a, b), again illustrating that this appendage forms without homeotic input.

To test whether the absence of *Antp* was important for wing formation, we examined wing discs in which *Antp* was ectopically expressed using the GAL4-UAS system¹⁴. Ectopic *Antp* expression had no effect on wing disc morphology or *vestigial* expression (not shown). Similarly, we examined the impact of *Scr* expression, which is normally absent from the wing disc, upon wing development. We find that wing development is sensitive to *Scr* repression in the embryo, but not at later stages. For instance, when *Scr* expression is driven ectopically in the growing wing disc, there is no apparent effect on wing formation as indi-

cated by disc morphology or *vestigial* gene expression (Fig. 2c). By contrast, ectopic *Scr* expression in the early embryo represses *vestigial* gene expression in the wing and haltere primordia (Fig. 2d, e).

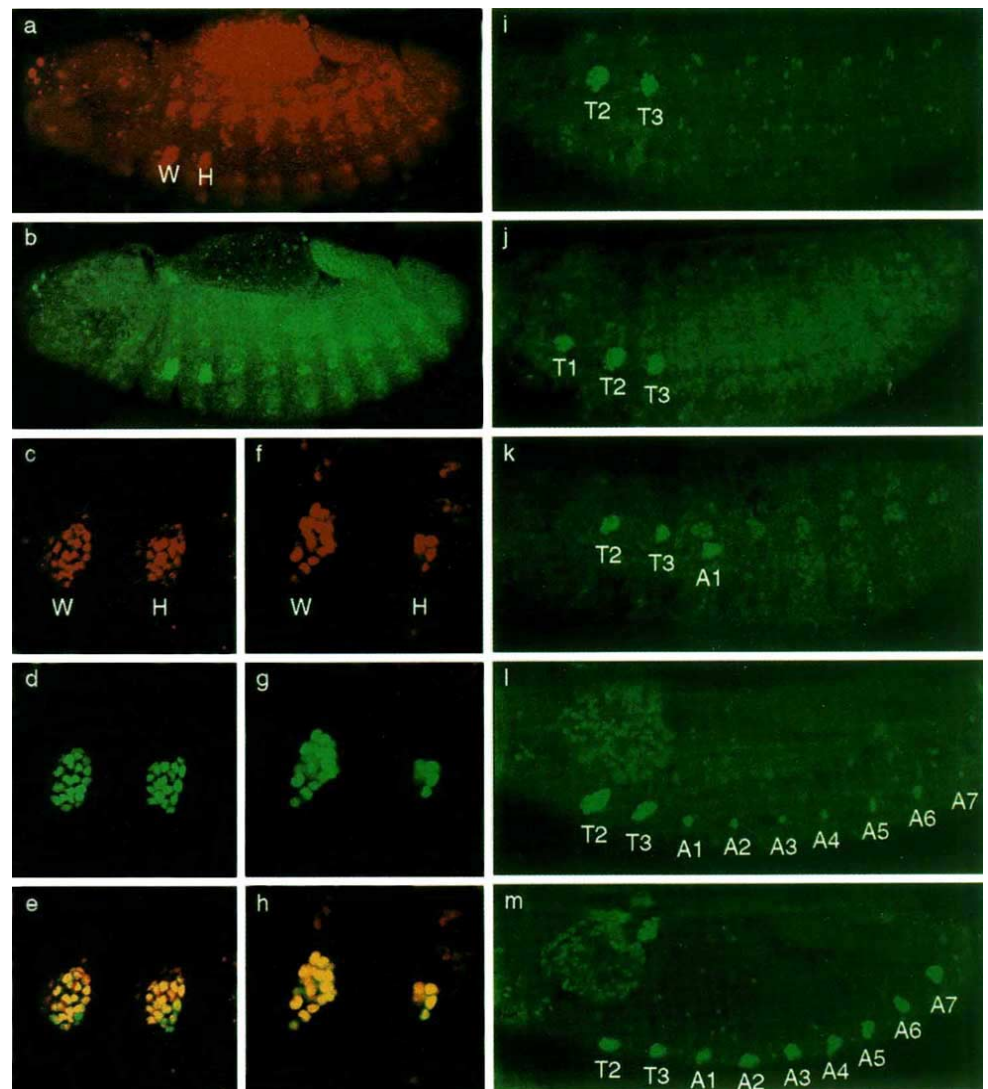
Fossil evidence indicates that wings or wing-like structures first appeared on all body segments of primitive pterygotes^{15,16} probably at first in the immature stages. This raises the question of how *Scr*, *Ubx* and *abd-A* came to repress wing formation in the course of pterygote and dipteran evolution. Two possible explanations are that these genes came into existence during pterygote evolution or that the homeotic genes diversified before pterygote evolution and homeotic regulation of wing number and type evolved subsequently. The presence of distinct *Scr*, *Ubx* and *abd-A* genes in crustacea¹⁷ suggests that the homeotic genes diversified before the insect/crustacean divergence and would each be present in a pterygote ancestor.

One way to address the issue of homeotic gene expression in pterygote ancestors is to examine their deployment in a primitive apterygote which would represent the condition before the 'invention' of wings. The Thysanura are the sister group of

pterygotes¹⁶ which have retained many primitive features. The *Ubx* and *abd-A* proteins¹⁸ are expressed in the posterior thoracic and anterior abdominal segments of the apterygote thysanuran *Thermobia domestica* (Fig. 3), as they are in pterygotes¹⁸. The similarity between apterygote and modern winged insects' BX-C protein patterns indicates that the spatial domains of these homeotic genes were probably established before the origin of wings and the diversification of the pterygotes. In primitive pterygotes that bore wings on abdominal segments, BX-C genes, although present and expressed in the abdomen, apparently did not regulate wing formation as they do in modern pterygotes.

We propose a plausible scenario for the role of homeotic genes in pterygote evolution that is based on the above developmental genetic studies of *Drosophila*, comparative analysis of homeotic gene expression and the fossil record (Fig. 4). When wings or their progenitors first arose and were found on each body segment, there was no homeotic gene input into their number or design (compare Fig. 4a and b). This is reflected by the absence of any requirement for *Antp* function in *Drosophila* embryos (Fig. 1i) or imaginal discs¹³ for the establishment of the wing

FIG. 1 Homeotic genes repress but do not promote formation of the flight appendage primordia in *Drosophila*. a, c, Stage-12 *Drosophila* embryo, about 20 *vestigial*-expressing cells mark the wing (W) and haltere (H) primordia, the rest of the pattern is largely in the muscle precursors; b, d, stage-12 *Drosophila* embryo, about 20 *snail*-expressing cells mark the wing and haltere primordia, the other labelled cells are in proneural clusters of the peripheral nervous system; e, stage-12 embryo double-labelled for *vg* and *sna* proteins, the patterns within the wing and haltere primordia are nearly or completely coincident; f, g, h, stage-17 embryo double-labelled for *vg*, (f), *sna*, (g), the patterns are coincident (h). The difference in the number of cells stained at the two stages is due to the restriction of gene expression to a subset of the cells within the developing disc. i–m, Wing and haltere primordia in stage-15 embryos homozygous for either the *Antp*^{W10}, *Scr*^{W17}, *Ubx*^{6,28}, *Ubx*¹⁰⁹ or *abd-A*^{Mx1} mutations detected by *snail* expression. i, Wing and haltere primordia are normal in an *Antp* mutant embryo; j, an additional primordium forms in the T1 segment of an *Scr* mutant embryo; k, the haltere primordia are enlarged and an ectopic primordium forms in a *Ubx* mutant embryo; l, a few *snail*-expressing cells arise in the posterior of segments A1–A7 in an *abd-A*[−] embryo, these cells stain because the wing primordia cross the parasegmental boundary and *Ubx* (which represses wing formation in the anterior compartment of A1–A7 in an *abd-A* mutant see (m)) is still repressed in the posterior compartment of segments A1–A7 in an *abd-A* mutant embryo by the *en* protein²¹; and m, wing primordia arise in segments T2–A7 in an *Ubx*¹⁰⁹ (*Ubx*[−], *abd-A*[−]) embryo. All embryos are magnified ×540 except c–h which are magnified ×1,600. METHODS. *Drosophila* embryos (OregonR) were stained with a rabbit anti-*vestigial* antibody and/or a monoclonal antibody to the *snail* protein²⁶ (gift from A. Alberga), an unconjugated goat anti-rabbit and/or a biotinylated donkey anti-mouse secondary antibody was added,



and either indocarbocyanine (Cy5)-conjugated streptavidin and/or a fluorescein-conjugated donkey anti-goat antibody were used as tertiary reagents. All embryos were visualized by confocal microscopy and mutant genotypes were confirmed by staining with antibodies against *Scr*, *Antp*, *Ubx*, or *Ubx/abd-A*.

FIG. 2 *Antp* and *Scr* do not influence imaginal wing outgrowth. **a**, Third instar imaginal disc stained with a monoclonal antibody to *Antp* which is expressed in a minor subset of anterior cells (purple), primarily in the future dorsal notum. **b**, Only a few cells of the thousands that will make up the future wing pouch and wing hinge (depicted here in green by *vestigial* expression) express the *Antp* protein (purple). **c**, UAS-*Scr* expression (red) driven along the A/P boundary by a *ptc*-GAL4 driver does not alter the level or pattern of *vestigial* expression (green) or the morphology of the growing third instar disc. **d**, *Scr* expression (purple) and *vestigial* expression (green) in a wild-type embryo; the wing and haltere primordia (are indicated by yellow arrows). **e**, The pattern of *Scr* expression in *Antp* P1GAL4- UAS; *Scr* embryo is shown is purple; *vestigial* expression (green) has been repressed where the wing and haltere primordia would normally arise (yellow arrows).

METHODS. Imaginal discs were stained as described in refs 27 and 28 and embryos as described in Fig. 1. UAS-*Scr*²⁹ expression in imaginal discs was driven with a *ptc*-GAL4 driver³⁰ and in embryos with an *Antp* P1-GAL4 driver²⁹.

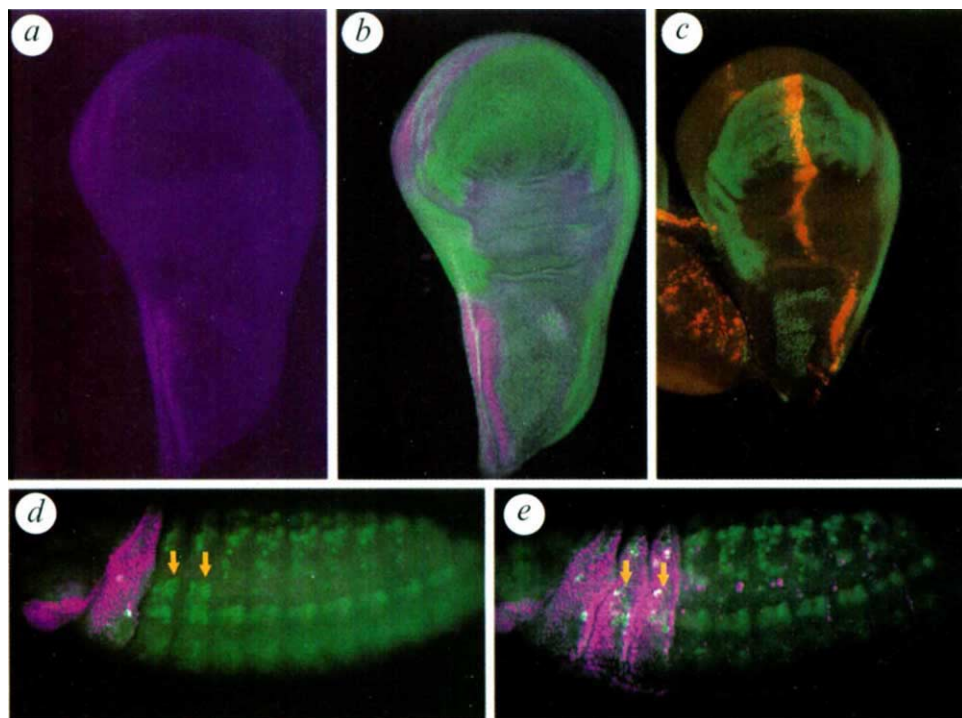
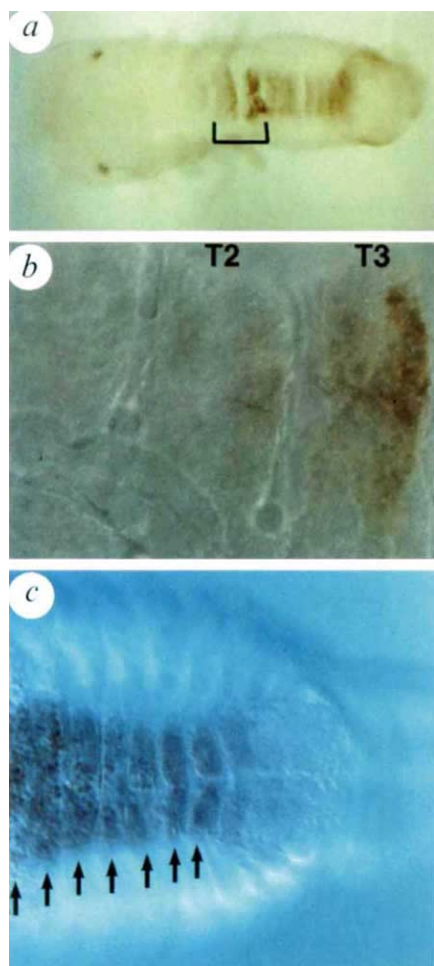


FIG. 3 BX-C proteins are deployed in the abdominal segments of a primitive apterygote insect (*Thermobia*). **a**, The *Ubx*/*abd-A* proteins are expressed in the posterior thorax and most of the abdominal segments, with the highest level of staining in T3 and A1 (bracket). Some of the gaps in staining, for example between T3 and A1 in the middle of the bracketed region, are due to curvature and buckling of the embryo, not the lack of antigen expression. **b**, A high-magnification view of the thorax reveals high levels of staining in the posterior of T3 and faint levels of staining in posterior T2. **c**, A high-magnification view of the abdomen reveals a contiguous pattern of expression from A1 to A7 (seven arrows). **METHODS.** *Thermobia domestica* embryos were incubated with an antibody that recognizes the *Ubx* and *abd-A* proteins¹³ of flies, locusts and butterflies, and horseradish peroxidase-conjugated goat anti-mouse secondary antibody and stained with diaminobenzidine.



primordia or morphogenesis of the wing. Subsequently, certain genes involved in wing formation must have evolved or acquired regulatory elements which responded to homeotic proteins. The ensuing modifications of the original pterygote wing or pro-wing pattern could have been achieved by the evolution of *Scr*-, *abd-A*- and *Ubx*-responsive *cis*-regulatory elements in genes involved in wing formation and/or through subtle changes in the expression domains of these homeotic repressors (for example, expression of *Scr* in T1 or modification of the *Ubx* pattern in T3). Assuming that the domains of homeotic gene expression have been conserved among insects (as suggested in part by Fig. 3), the evolution of *Scr*-responsive elements led to the modification (Fig. 4d) or elimination (Fig. 4c) of prothoracic wings and the evolution of *abd-A*- and *Ubx*-responsive elements led to the elimination of abdominal wings (Fig. 4d) and, in the Diptera, the reduction of metathoracic wings (Fig. 4f).

The diversification of the pterygotes provides an example of how a new structure, once invented independently of the homeotic genes, may be modified by homeotic genes in the course of evolution. It is possible that the evolution of other body plans has followed a similar sequence of invention followed by homeotic modification or reduction of a character. The formation of trunk limb primordia in *Drosophila* is also under the negative

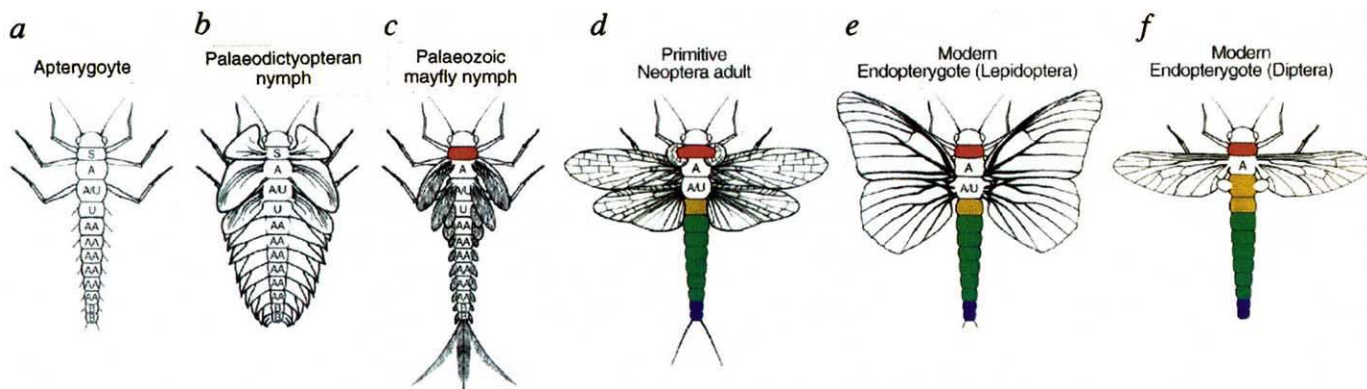


FIG. 4 Homeotic genes and pterygote evolution. A highly stylized view of pterygote evolution from an apterygote ancestor (a) to various extinct orders (b–d) to modern Lepidoptera (e) and Diptera (f). The fossil evidence upon which a–d are based is from Kukalova-Peck^{1,2} and specimens described by Wootton¹⁵. The segmental body plan of each animal is represented as identical body designs and the homeotic genes expressed in different segments are represented by letters (S, Scr; A, Antp; U, Ubx; AA, abd-A; B, abd-B). The segments in which individual

homeotic genes are postulated to regulate wing formation are indicated by colouring in the respective segments at the appropriate stage of pterygote evolution (Scr, red; Ubx, yellow; abd-A, green). Note that *Antp* is never depicted as regulating wing formation. In the palaeodictyopteran nymph (b), the homeotic 'groundplan' was fully diversified as in modern insects but wings formed on all segments. In four-winged insects (e) *Ubx* represses wing formation in A1 and on two-winged insects (f) in T3 as well.

control of homeotic genes^{19,20}, and as in wing formation, *Antp* is not required for limb formation²¹. It is possible that the evolution of insects from a myriapod-like ancestor²² involved the evolution of homeotic repression of limb formation, rather than the evolution of new homeotic genes, as has been postulated^{23–25}. The evolution of regulatory interactions between homeotic proteins and genes involved in appendage formation and of changes in homeotic gene regulation may then be the key steps in the diversification of arthropod body plans and appendages. □

Defective axonal transport in a transgenic mouse model of amyotrophic lateral sclerosis

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AMYOTROPHIC lateral sclerosis (ALS) is a degenerative disease of motor neurons, characterized by depositions of neurofilaments in the perikarya and proximal axons. The pathogenesis of ALS remains poorly understood, but two lines of evidence suggest that neurofilament accumulation may play a causal role. First, transgenic mice that overexpress neurofilament proteins show motor neuron degeneration^{1–3} and, second, variant alleles of the neurofilament heavy-subunit gene (*NF-H*) have been found in some human ALS patients⁴. To investigate how disorganized neurofilaments might cause neurodegeneration, we examined axonal transport of newly synthesized proteins in mice that overexpress the human *NF-H* gene¹. We observed dramatic defects of axonal transport, not only of neurofilament proteins but also of other proteins, including tubulin and actin. Ultrastructural analysis revealed a paucity of cytoskeletal elements, smooth endoplasmic reticulum and especially mitochondria in the degenerating axons. We therefore propose that the neurofilament accumulations observed in these mice cause axonal degeneration by impeding the transport of components required for axonal maintenance, and that a similar mechanism may account for the pathogenesis of ALS in human patients.

The motor dysfunction in mice with a modest overexpression of the human *NF-H* transgene (line 200) (ref. 1) progresses during ageing by the atrophy and subsequent degeneration of axons distal to the neurofilament swellings. Light microscopy examination of the L5 ventral roots from a 2-year-old *NF-H* transgenic mouse revealed a massive degeneration of large axons derived from spinal motor neurons (Fig. 1).

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To clarify the mechanism of neurodegeneration in *NF-H* transgenics, we examined whether intracellular transport of axonal proteins was impaired. [^{35}S]Methionine was injected into the spinal cord and, after different time intervals, the profiles of ^{35}S -labelled proteins in 5-mm segments of the sciatic nerve were analysed by fluorography of SDS-polyacrylamide gels as described previously⁵. Figure 2 shows fluorographic patterns of radiolabelled proteins transported in the sciatic nerve at 7 and 28 days after injection of [^{35}S]methionine into the spinal cord of normal mice and *NF-H* transgenic mice. In normal mice (Fig. 2a, b), the NF-L, NF-M and NF-H proteins move into motor axons as a triplet with a velocity of $\sim 0.5 \text{ mm d}^{-1}$, in agreement with previous reports^{5,6}. In motor axons of *NF-H* transgenics, there is a remarkable reduction in the rates of transport of neurofilament proteins. In 3-month-old transgenics, all these neurofilament subunits moved into motor axons at a decreased rate (Fig. 2c, d). Note the anomalous stoichiometry of transported neurofilament proteins and the exceedingly low levels of NF-M protein (Fig. 2d).

The impairment of axonal transport in motor neurons of *NF-H* transgenic mice is not limited to neurofilament proteins. As shown in Fig. 2, there are transport defects for tubulin and actin proteins, two cytoskeletal proteins of the slow-moving components. In normal mice, 28 days after injection of [^{35}S]methionine into the spinal cord, the peaks of incorporated radioactivity for tubulin and actin proteins occurred in the 10-mm segments of the sciatic nerve, respectively (Fig. 2b). In contrast, for the *NF-H* transgenics of 3-month-old mice, the peaks of tubulin and actin remained in the proximal 5-mm segment of the sciatic nerve

(Fig. 2d). The observed defects for tubulin and actin transport are less substantial than those observed for neurofilament proteins. This is not surprising, however, because the experimental approach did not discriminate between tubulin and actin proteins migrating into large, neurofilament-rich axons of the sciatic nerve from those migrating into small, surviving axons.

Electron microscopy confirmed a general depletion of crucial organelles in atrophied axons of the sciatic nerve from 3-month-old *NF-H* transgenic mice. As shown in the longitudinal sections in Fig. 3, when compared to normal axons of similar myelin thickness (Fig. 3a), shrunken transgenic axons (Fig. 3b) are clearly deficient in filamentous structures, mitochondria and small endoplasmic reticula. The lack of mitochondria in degenerating motor axons of *NF-H* transgenics was also confirmed (Fig. 4) by the weak immunofluorescence staining of longitudinal sections of L5 ventral roots (Fig. 4b) with an antibody specific to the inner membrane of the mitochondrion (monoclonal MOM/H6/C12 from Serotec). This anti-mitochondrion antibody yielded an intense staining of L5 ventral root axons from normal mice (Fig. 4a). The immunofluorescence staining of spinal cord sections from *NF-H* transgenic mice revealed the trapping of mitochondria within the perikaryon of spinal motor neurons (Fig. 4d). The mitochondria in degenerating neurons were mainly detected near the nucleus, surrounded by large cytoplasmic aggregates of neurofilaments.

The abnormal accumulation of neurofilaments in motor neurons is typical of ALS, occurring in about 70% of ALS cases^{7,8}. The results presented here indicate that the accumulation of neurofilaments can play a central role in motor neuron degeneration.

a

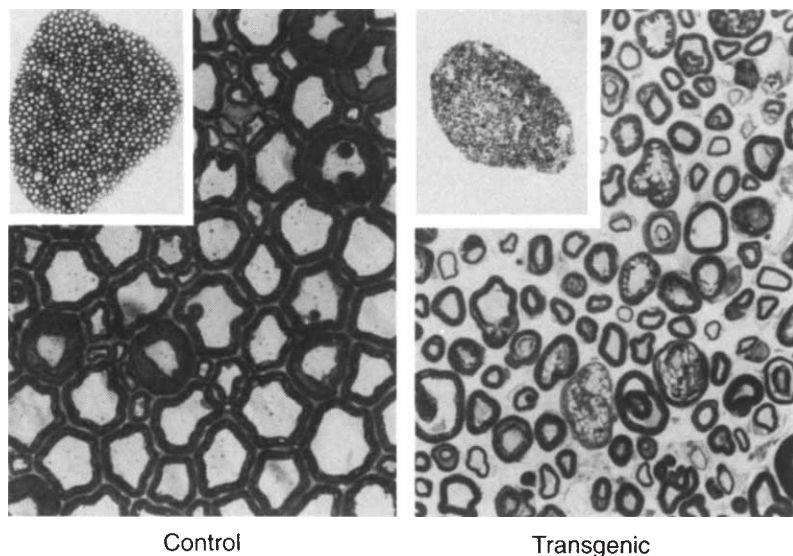


FIG. 1. Motor-axon degeneration in L5 ventral root of *NF-H* transgenic mice. a, Light-microscopic examination of cross-sections of L5 ventral roots from normal (left) and *NF-H* transgenic (right) mice at 2 years of age. The insets show sections of the entire roots. Magnifications $\times 920$; insets, $\times 80$. b, Selective loss of large motor axons in L5 ventral root.

METHODS. Mice were anaesthetized with nembutal and perfused using 0.9% NaCl, 2.5% glutaraldehyde, 0.5% paraformaldehyde in sodium phosphate buffer, pH 7.4. Ventral root samples were immersed in fixative for 2 h, rinsed in phosphate buffer and postfixed for 1.5 h in 1% osmium tetroxide. After 3 washes with phosphate buffer, each sample was dehydrated in a graded series of ethanol and embedded in Epon. The thin sections were stained with toluidine blue and examined under a Nikon Labophot microscope.

b

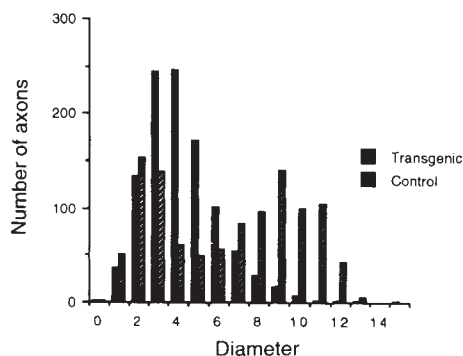
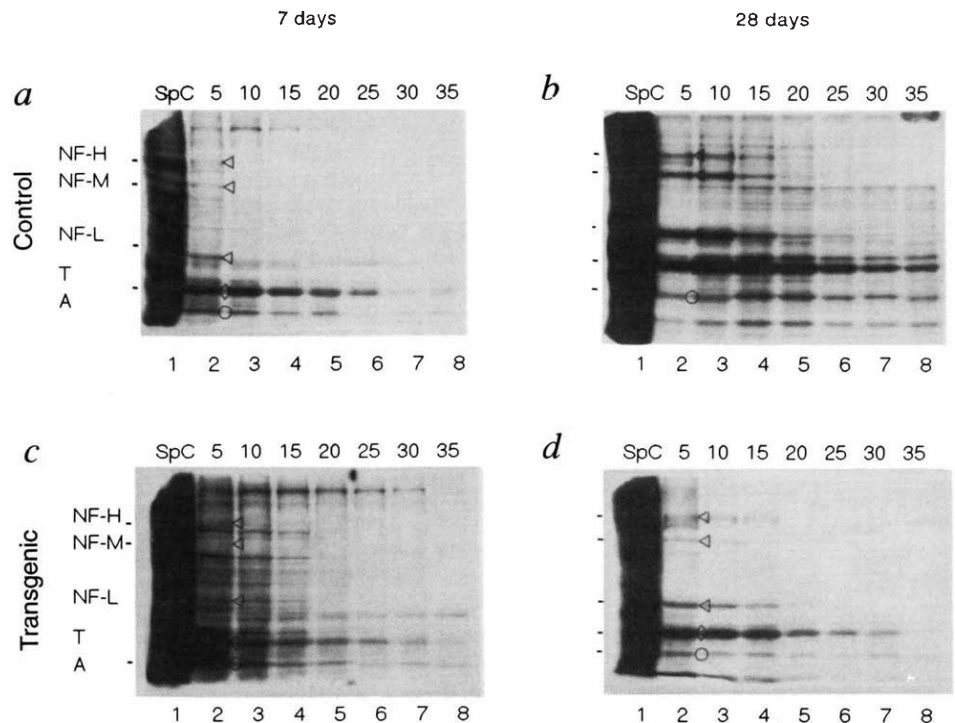


FIG. 2 Fluorographs of slow transport profiles in motor axons of the sciatic nerve from normal and *NF-H* transgenic mice. *a, b* Profiles of normal 3-month-old mice 7 and 28 days after intraspinal injection of [35 S]methionine. *c, d* The corresponding results from 3-month-old *NF-H* transgenic mice. For each panel, lane 1 contains proteins extracted from the site of injection (L4–L5 spinal cord), and each successive lane represents a 5-mm nerve segment extending distally to the right. Note the reduced rate of transport for all three neurofilament (NF) subunits in transgenic mice (*c* and *d*). Transport defects are also evident for actin and tubulin in transgenic mice. In normal mice, after 28 days pulse-labelling, the peaks for tubulin and actin proteins are found 10 mm and 15 mm away from the spinal cord (*b*), whereas the peaks for these two proteins in transgenic mice are detected in the 5-mm segment (*d*).

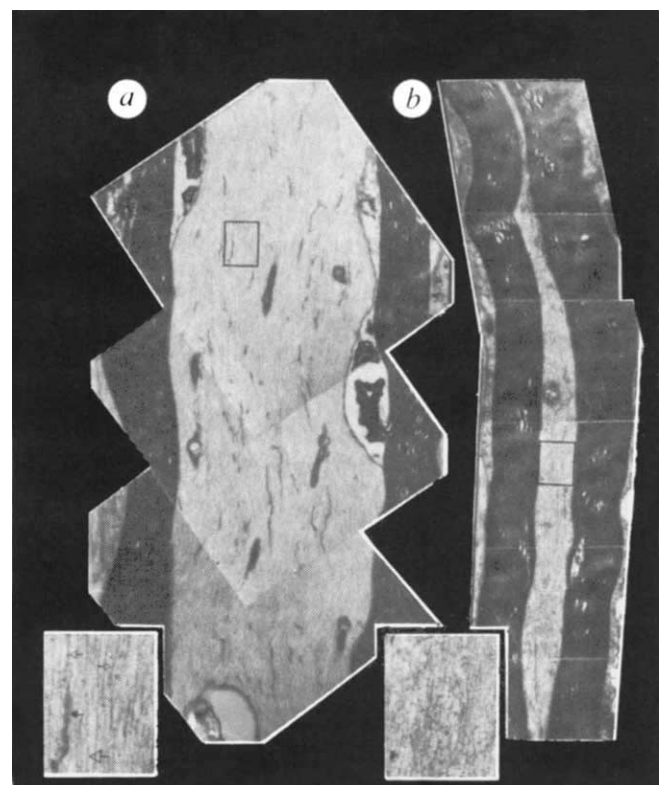
METHODS. Homozygous C57Bl/6j transgenic mice from line 200 and a control mouse (all 3 months old) were used. Mice were anaesthetized with nembutal. The lumbar spinal cord was exposed by laminectomy, and newly synthesized proteins in motor axons in the sciatic nerve were labelled by the injection of a total of 3 μ l [35 S]methionine (Amersham; specific activity $>1,000$ Ci mmol $^{-1}$) through a glass micropipette connected to a Hamilton syringe filled with mineral oil, into each of 3 sites of the right anterior horn of the lower lumbar enlargement (L4–L5). Cells in that region give rise to motor axons of the sciatic nerve. The isotope was dried down and resuspended in distilled water to a final concentration of 150 μ Ci μ l $^{-1}$ before injection. The mice were killed 7 and 28 days later. The right L4–L5 ventral root and sciatic nerve were removed in continuity, cut into 5-mm segments and homogenized in 115 μ l SUB buffer (0.5% SDS, 8 M urea, 2% β -mercaptoethanol). Homogenates were



centrifuged at 10,000 r.p.m. in a table-top centrifuge. A 10- μ l portion was analysed using 8% SDS-PAGE (polyacrylamide gel electrophoresis). Gels were then stained with Coomassie blue, destained in methanol/acetic acid, treated with Amplify (Amersham), dried and processed for fluorography. The labelled proteins on the gels were visualized by exposing dried gels to Kodak XAR films at -80°C for 2 to 14 days. Each lane represents a pool from the sciatic nerves of 3 animals. The same results were obtained from 2 independent experiments.

FIG. 3 electron microscopy of longitudinal sections of myelinated axons from the sciatic nerve of normal and *NF-H* transgenic mice. Note the depletion of filaments, microtubules, mitochondria and smooth endoplasmic reticulum in the shrunken myelinated axon of *NF-H* transgenic mice (*b*) as compared with a normal axon of similar myelin thickness (*a*). Small open arrows show microtubules in a normal axon. The large arrow points to a neurofilament, and the small filled arrow indicates a smooth endoplasmic reticulum. Magnification $\times 4,370$; insets, $\times 14,420$.

METHODS. Electron-microscopic analysis. The samples were prepared from 3-month-old mice as described in Fig. 1. Ultrathin sections of the sciatic nerve were stained for 8 min in lead citrate and examined with a Philips CM10 electron microscope.



tion by disrupting the intracellular supply of components required for axonal integrity. In particular, there is a deficient transport of mitochondria into motor axons distal to the neurofilament swellings. A shortage in mitochondria might be expected to cause a severe disturbance in energy metabolism, resulting in a neuropathy. Such a 'dying back' model of nerve degeneration has been proposed as a plausible mechanism for ALS⁹⁻¹¹. A disruption of axonal transport by neurofilament disorganization is a pathological mechanism consistent with several aspects of ALS. First, it provides an explanation for the cellular selectivity of the disease. Large motor neurons are particularly vulnerable to neurofilament abnormalities because of their high level of synthesis of neurofilament proteins¹². Second, there is a retardation in the slow axonal transport of cytoskeletal elements during ageing¹³, a factor that can predispose to the disease. Third, involvement of neurofilaments in ALS pathogenesis is compatible with the finding of neurofilament accumulation in motor neurons of some familial ALS cases induced by mutations in the superoxide dismutase gene *SOD-1* (cited in ref. 3), and with reports of aberrant neurofilament swellings in degenerating motor neurons of transgenic mice that express a mutant form

of human *SOD-1*¹⁴. The discovery that abnormal neurofilament accumulations can contribute to motor neuron degeneration provides a basis for developing therapeutic approaches for ALS. Attempts to reduce the neurofilament swellings, which depend on the discovery of drugs capable of downregulating neurofilament expression, might help to slow down the devastating course of this disease. □

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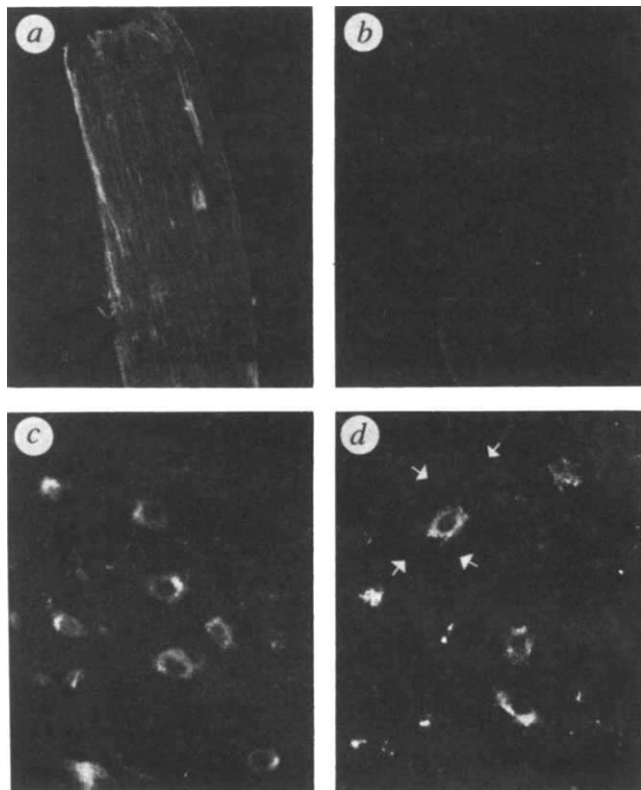


FIG. 4 Immunofluorescence detection of mitochondria in ventral roots and spinal motor neurons. The ventral root axons from an *NF-H* transgenic mouse yielded a weak anti-mitochondria staining (b) as compared with those from a normal mouse (a). Magnification $\times 72$. c, Mitochondria are detected throughout the cytoplasm in normal spinal motor neurons. d, In degenerating motor neurons of *NF-H* transgenics, the mitochondria, which are surrounded by large NF aggregates, are trapped near the nucleus. The arrows point to cell boundaries. c and d, Magnification $\times 286$.

METHODS. Immunofluorescence microscopic analysis. Anaesthetized mice were perfused with 0.9% NaCl and 4% paraformaldehyde in sodium phosphate buffer, pH 7.4. Sciatic nerves were rinsed in phosphate buffer and immersed in 15% sucrose and phosphate buffer. Cryostat sections (10 μ m) were first incubated with anti-inner-membrane mitochondria antibody (Serotec MOM/H6/C12) and then incubated with anti-mouse biotin (Jackson Laboratories), and fluorescein-labelled streptavidin. Sections were examined under a Nikon Labophot microscope.

Tumorigenesis and metastasis of neoplastic Kaposi's sarcoma cell line in immunodeficient mice blocked by a human pregnancy hormone

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KAPOSI'S sarcoma (KS) occurs more often in men than in women and HIV-1-associated KS has a high occurrence in homosexual men (over 30%). Most cultures of KS tumours yield cells with properties of hyperplastic (not malignant) endothelial cells under the control of several cytokines¹⁻⁷. The role of HIV-1 may be in promoting high levels of some cytokines and providing stimulation to angiogenesis by the HIV-1 Tat protein⁸, which synergizes with basic fibroblast growth factor in promoting these effects⁹. Here we describe an immortalized AIDS-KS cell line (KS Y-1) and show that these cells produce malignant metastatic tumours in nude mice

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TABLE 1 Inhibition of KS Y-1 growth in clonogenic *in vitro* assays**(a) Inhibition by sera of pregnant mice and humans**

Cells	Source of sera	None	Sera (% v/v)						
			1%	Early pregnancy		10%	1%	Late pregnancy	
				5%			5%	10%	
			Mean number of colonies						
KS Y-1		191							
	Mouse (<i>n</i> = 3)		37	6	0	141	105	69	
	Human (<i>n</i> = 4)		27	11	3	167	121	79	
			Sera from non-pregnant individuals or mice						
			1%	5%	10%				
KS Y-1	Male (human)		193	178	179				
	Non-pregnant female (human)			177	170	175			
C10 MJ		309							
	Mouse		283	299	304	298	294	310	
	Human		275	279	308	300	301	297	

(b) Inhibition of growth by hCG and the beta chain of hCG

Cells	None	Treatment ($\mu\text{g ml}^{-1}$)					
		hCG 12.5 $\mu\text{g ml}^{-1}$	1 $\mu\text{g ml}^{-1}$	βhCG 10 $\mu\text{g ml}^{-1}$	1 $\mu\text{g ml}^{-1}$	αhCG 10 $\mu\text{g ml}^{-1}$	Number of colonies
KS SLK	218	15	20	0	210	183	
KS Y-1	234	10	24	0	224	179	
C10 MJ	288	273	291	283	280	289	
PBMC	320	319	287	290	318	330	

a, The KS Y-1 cell line originated from mononuclear cells isolated from 2.5 l of pleural effusion of an AIDS patient with KS involving the lungs. After the depletion of T lymphocytes, monocytes/macrophages and fibroblasts by the cytotoxicity method, using monoclonal antibodies against CD2, CD3, CD4, CD8, CD10 and CD14 membrane antigens and baby rabbit complement, the cells were cultured in the absence of exogenous growth factors in an attempt to select for transformed cells. The KS Y-1 cells have been maintained for over 100 passages and, therefore, may be appropriately designated a cell line. More extensive immunological characterization by FACScan and immunofluorescence assays showed that CD34, CD31 and endoglin were expressed by KS Y-1 (our unpublished results), suggesting an endothelial cell origin. Similar results were found in a case of classical (non-HIV-1-associated) KS, although these cells (KS SLK)¹⁰ did not metastasize (our unpublished results). The growth of KS Y-1 and KS SLK was not dependent on added growth factors. KS Y-1 cells were seeded in methylcellulose (0.8%, v/v). Clonogenic assays were done in the presence of an increasing amount of sera (0%, 1%, 5% or 10% v/v) from pregnant women or mice. Colonies (>50 cells) were counted after a 10-day incubation period. The number of colonies is the mean from triplicate wells after seeding 5×10^4 cells per culture in 0.1 ml media. The s.d. did not exceed 10% of the mean values. C10 MJ is an immortalized human adult T-cell line¹². b, KS Y-1, KS SLK, human neoplastic T-cells, or normal peripheral blood PHA-stimulated T-cells ($5 \times 10^5 \text{ ml}^{-1}$) were seeded in methylcellulose (0.8%, v/v). Clonogenic assays were done in the absence or presence of native hCG (hCG from Sigma (CG-10) and Wyeth Ayerst Lab (APL)), βhCG or αhCG . Results are expressed as the mean number of colonies of triplicate wells formed after seeding with 5×10^4 cells. s.d. was less than 10% of the mean values. C10 MJ is an immortalized human T-cell line¹².

PBMC, PHA-activated normal peripheral blood mononuclear cells were stimulated with PHA-P ($5 \mu\text{g ml}^{-1}$) and interleukin-2 (10 IU ml^{-1}) for 3 days¹¹.

and are killed *in vitro* and *in vivo* (apparently by apoptosis) by a pregnancy hormone, the β -chain of human chorionic gonadotropin. Similarly, chorionic gonadotropin kills KS SLK, cells from another neoplastic cell line (established from a non-HIV-associated KS)¹⁰, as well as the hyperplastic KS cells from clinical specimens grown in short-term culture, but does not kill normal endothelial cells. These results provide evidence that KS can evolve into a malignancy and have implications for the hormonal treatment of this tumour.

Both KS Y-1 (see legend to Fig. 1 for methods) and KS SLK¹⁰ have abnormal chromosomes and produce malignant tumours in immunodeficient mice (Fig. 1) in contrast to all other cultured cells from KS specimens, which grow transiently in culture and do not produce neoplastic lesions. Because KS is more frequent in males and was noted to regress in pregnancy, we hypothesized that one or more hormones might be involved. Four female and two male neonatal immunodeficient Bg-nude mice (caged together) were inoculated intraperitoneally (i.p.) and twenty female and nineteen male adult mice subcutaneously (s.c.) with KS Y-1 cells. All adult mice and the neonatal males developed malignant metastatic tumours 1 month after inoculation. However, the four female litter mates who became pregnant remained

tumour free. KS Y-1 cells were then inoculated into mice in early- and late-stage pregnancy. Mice inoculated during the early stage of pregnancy (days 1–10; average gestation is 19 days) did not develop tumours. Inoculation during the latter stages (days 13–21) generated tumours of reduced size which did not metastasize (data not shown). Typical metastatic tumours developed in all non-pregnant mice.

In vitro proliferation and clonogenic assays¹¹ were done using KS Y-1 cells in the presence of sera obtained from humans and mice during the early and late stages of pregnancy (Table 1a). There was a marked inhibition of growth with sera obtained during the early stages of pregnancy, minimal inhibition with sera from later stages, and no effect of sera from non-pregnant mice and humans. Also, no sera effected growth of human T cells (Table 1a) or normal human umbilical vein endothelial cells (HUVEC) (data not shown). Because human chorionic gonadotropin (hCG) levels are higher during early pregnancy, we tested the effects of purified native hCG (containing 80% βhCG and 20% αhCG), native αhCG , and native βhCG on KS Y-1 growth (Table 1b). αhCG had little inhibitory effect, whereas both native hCG and βhCG inhibited KS Y-1 cell growth and the effect was dose-dependent. Similarly, growth of

TABLE 2 *In vitro* or *in vivo* pretreatment of immunodeficient mice (B2-nude) with hCG inhibits tumorigenesis induced by KS Y-1 cells**(a) *In vitro* treatment**

	Tumour size (mm)	Angiogenesis	Metastasis
hCG source A (n = 6)	0*	0	0
hCG source B (n = 6)	0*	0	0
No HCG	17×22 to 27×30	+	+

(b) *In vivo* treatment

	Tumour size (mm)	Angiogenesis	Metastasis
None	45×35	+	+
	41×19	+	+
	42×39	+	+
	39×37	+	+
hCG source A	0×0	NA	NA
	0×0	NA	NA
	0×0	NA	NA
	2×3	+	—
	1×2	+	—
	2×2	+	—
hCG source B	0×0	NA	NA
	0×0	NA	NA
	0×0	NA	NA
	1×1	+	—
	1×1	+	—
	2×1	+	—

a (*in vitro*), KS Y-1 cells (5×10^5) were treated for 48 h with control (PBS) or 100 IU ml⁻¹ hCG from source A (Sigma: CG-10) or source B (Wyeth Ayerst Lab: APL) before s.c. injection into Bg-nude mice. Animals were killed 6 weeks post-inoculation. b (*in vivo*), Animals were pretreated with hCG 100 IU (i.p. or s.c.) daily for 1 week and then injected s.c. with KS Y-1, 10^6 cells per 0.1 ml. The mice were examined for the presence of tumours, angiogenesis and metastasis 3–4 weeks post-inoculation. Source A (Sigma: CG-10) was inoculated s.c. whereas source B (Wyeth Ayerst Lab: APL) was inoculated i.p. The experiment was repeated 5 times (with 6 mice per group). The data shown are representative samples.

n, Number of animals of test group.

* No tumours were observed by macroscopic or light microscopic evaluation of the injection site 6 weeks post-injection.

NA, Not applicable.

KS SLK and cell strains KS 1510 and KS-4¹ was blocked in standard culture as measured by thymidine incorporation and Trypan blue exclusion, but there was little or no effect on growth of HUVEC, or human T cells (cell line C10 MJ¹² and phytohaemagglutinin (PHA)-stimulated peripheral blood T cells) (data not shown).

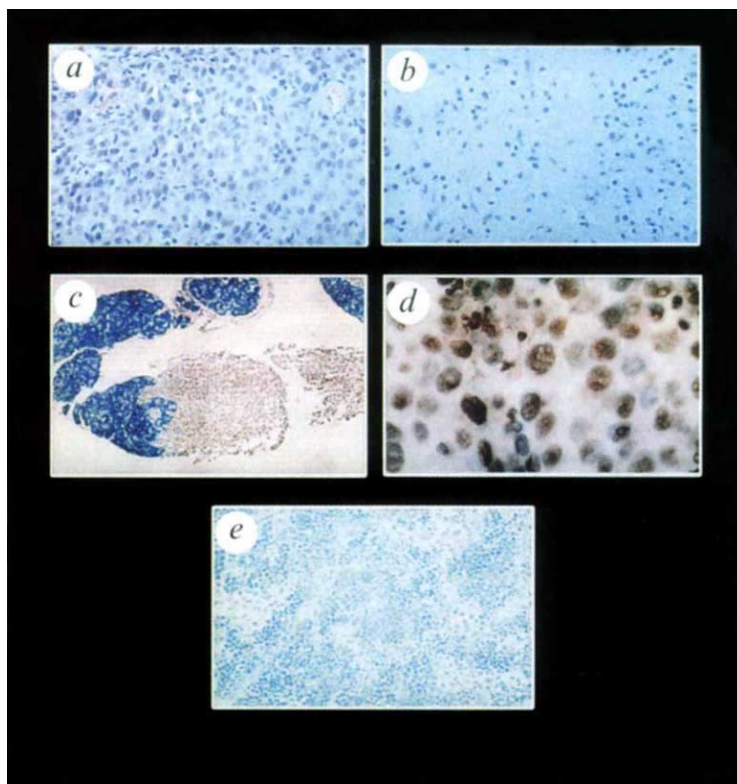
KS Y-1 cells were pretreated *in vitro* for 48 h with 100 IU ml⁻¹ (12.5 µg ml⁻¹) hCG and then inoculated i.p. or s.c. into immunodeficient mice (Table 2). All mice inoculated with untreated cells developed metastatic tumours, whereas no tumours occurred in mice inoculated with cells pretreated with hCG. Daily administration of hCG (100 IU per day) to mice for 1 week followed by inoculation of cells markedly inhibited tumour development (Table 2).

Histological examinations of the KS Y-1 tumours show many mitotic figures (Fig. 1a). Necrosis is evident after treatment with 100 IU per day of hCG for 7 days (Fig. 1b, c). Dense nuclear masses (Fig. 1b) and DNA fragmentation assays^{13,14} (DNA 3'-OH termini) (Fig. 1d) suggest that the effect of hCG involves cell killing by apoptosis.

It is important to know whether cells from primary KS specimens also respond to hCG. KS cells obtained from pleural effusions were grown in short-term culture. Like the immortalized KS cell lines, these cells (KS-4, KS N1512, KS N1540, KS N1544, KS N1542) were also killed by hCG (not shown). In other experiments we detected a band of M_r 70K using radio-labelled β hCG chemically crosslinked to the cell membrane of the short-term cultured KS cells and KS Y-1. The band coincides with the M_r of β hCG and its receptor¹⁵ (Fig. 2A). As anticipated, the alpha and beta chain bands of hCG were absent when the crosslinking studies were done in the presence of 1,000-fold excess of unlabelled β hCG (Fig. 2B). In addition, all biopsy specimens from primary lesions of AIDS-KS patients (5 of 5) were positive whereas normal human skin was negative for hCG receptors by immunohistochemical staining (Fig. 2C). These results suggest that cell killing by β hCG may be due to a direct interaction with its receptor.

The low rate of KS in women might be due to the interplay of hormones in the regulation of vascular proliferation¹⁶. A

FIG. 1 Tumours induced in immunodeficient mice with KS Y-1 cells undergo apoptosis when treated with hCG. a, A thin tissue section of KS Y-1 tumour (untreated) shows the presence of frequent mitotic figures. b, A thin tissue section of tumour after the mouse was treated s.c. with hCG (100 IU ml⁻¹) daily for 7 days. There are areas with a small number of cells still remaining. Some contain dense nuclear masses. c, A metastatic tumour of pancreas in treated animals with hCG. d, Treated and e, untreated tumours of mice. c–e, The samples were stained *in situ* for the presence of cells with DNA fragmentation. Tissue slides from formalin-fixed tumours were treated with terminal deoxynucleotidase for extension of DNA ends (hydroxyl 3') and incorporation of digoxigenin-11-dUTP according to the manufacturer's instructions (Oncor, Gaithersburg, MD). Anti-digoxigenin antibody conjugated with the enzyme peroxidase allows detection of apoptotic cells that stain brown whereas viable cells stain blue.



phenomenon similar to our laboratory experimental results was observed by two of us (P.H. and J.M.B.) in HIV-positive pregnant women. A 35 year old African woman with a 2-year history of AIDS-KS (observation by P.H.) (cutaneous lesions, $CD4^+$ cells: 61 mm^{-3}), became pregnant and 5 months into her pregnancy none of 14 KS lesions progressed, and within 5 months of delivery of a KS-free, HIV-1 seropositive child, all lesions disappeared. The second woman, a 27 year old Caucasian intravenous drug user with a 6-month history of AIDS-KS (5 KS lesions, $CD4^+$ cells: 490 mm^{-3}), became pregnant and within the first 2 months of pregnancy all of the observed KS lesions disappeared. Among the hormones which appear in early pregnancy, βhCG is the most predominant^{17,18}. βhCG levels in pregnant women range from 160 IU ml^{-1} during the first trimester to under 10 IU ml^{-1} for the remainder of pregnancy¹⁹⁻²¹. hCG

belongs to the family of related hormones which include luteinizing hormone (LH), follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH)²¹. The hCG molecule consists of two non-covalently linked glycosylated polypeptide subunits, α and β ¹⁸. The α -subunits of hCG, LH, FSH and TSH are identical, whereas the β -subunit of hCG is 85% homologous to βLH and 35% homologous to βFSH ^{22,23}. Though there is a functional equivalent of hCG in sera of pregnant mice²⁴, the protein has not been identified. Our results suggest that the putative murine protein may have structural similarity to hCG. The similarity in amino-acid sequence of βhCG to βLH , a hormone also present cyclically at high levels in non-pregnant females, suggests that the cycling levels of hormones during menstruation might offer control over neovascularization^{16,25}, and may contribute to the lower incidence of KS in women.

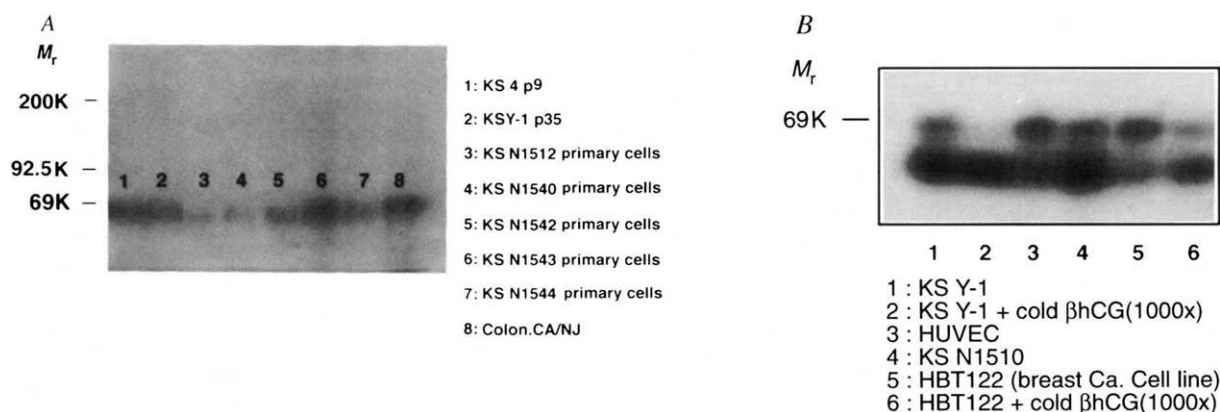
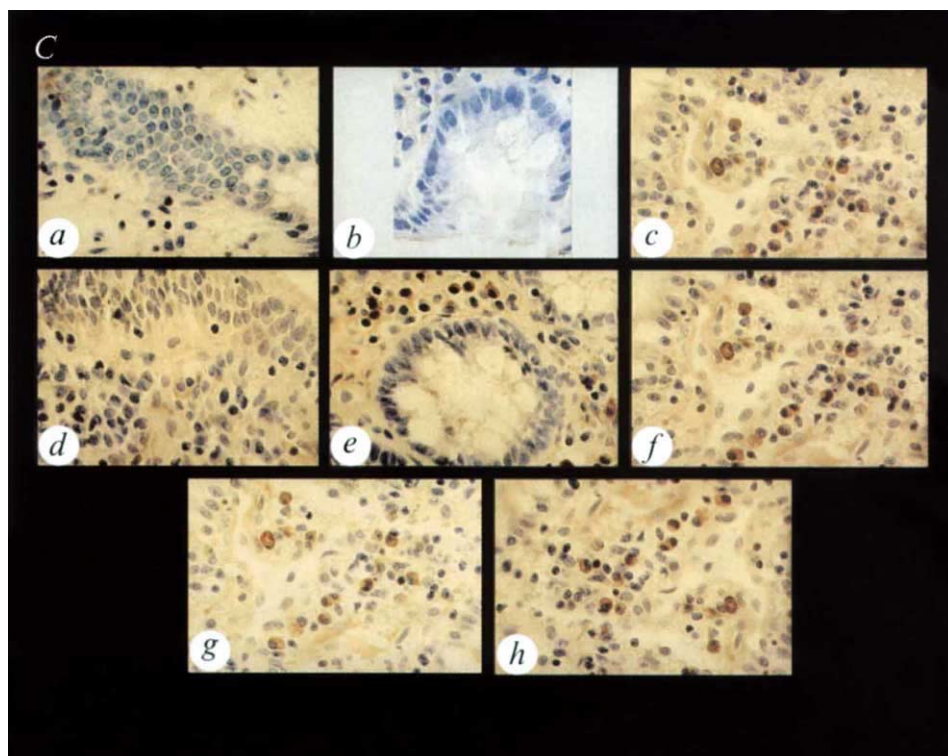


FIG. 2 A, Chemical crosslinking of hCG receptors with ^{125}I - βhCG in primary KS cells, a KS cell strain (KS-4), and the KS Y-1 cell line: lane 1, KS cell strain (KS-4, passage 9); lane 2, KS Y-1 cell line (passage 35); lanes 3-7, primary cells from AIDS-KS patients (KS N1512, KS N1540, KS N1542, KS N1543 and KS N1544); and lane 8, colon carcinoma cells (patient N1539). Binding of ^{125}I - βhCG and ^{125}I -hCG were as described²⁶. B, Binding of ^{125}I -hCG was done in the absence or in the presence of 1,000-fold excess of unlabelled βhCG ²⁶: lane 1, KS Y-1 cell line; lane 2, KS Y-1 + a 1,000-fold excess of unlabelled βhCG ; lane 3, HUVEC; lane 4, KS 1510; lane 5, HBT 122 (a breast carcinoma cell line); and lane 6, HBT 122 + a 1,000-fold excess of βhCG . Cells (5×10^6) were incubated for 10 min at 4°C with $1\text{ }\mu\text{l}$ of 1 mg ml^{-1} disuccinimidyl suberate solution (DSS) in dimethyl sulfoxide (DMSO). The reaction was stopped by quenching the unreacted DSS with $10\text{ }\mu\text{l}$ of a 1 mM ammonium acetate solution. After 1 min the cells were washed twice at 4°C with 10 mM Tris-HCl, 0.15 M NaCl containing EDTA. The ^{125}I -hCG pulsed, DSS crosslinked cells were extracted in 0.5% NP40 in 0.05 M Tris-HCl, 0.15 M NaCl containing $50\text{ }\mu\text{g ml}^{-1}$ aprotinin, $50\text{ }\mu\text{g ml}^{-1}$ leupeptin, 0.1% sodium azide and 10 mM sodium pyrophosphate at 4°C for 30 min. The lysates were clarified by centrifugation ($15,000\text{ r.p.m.}$ for 15 min at 4°C) and size fractionated in a 10% SDS-PAGE gel. C, Embedded KS tissue sections from AIDS-KS patients and from normal human skin were fixed in cold acetone and double-stained with a polyclonal antibody



to hCG using a peroxidase-anti-peroxidase staining method. Non-KS skin: a, b and d (donors: a with dermatitis, and b and d, normal donors). KS skin lesions: c, e-h.

To our knowledge, this is the first demonstration of an anti-tumour property of β hCG, and offers a new strategy for treating patients with Kaposi's sarcoma. □

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Nitric oxide triggers a switch to growth arrest during differentiation of neuronal cells

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ARREST of cell division is a prerequisite for cells to enter a program of terminal differentiation. Mitogenesis and cytotaxis of neuronal cell precursors can be induced by the same or by different growth or trophic factors^{1–9}. Response of PC12 cells to nerve growth factor (NGF) involves a proliferative phase that is followed by growth arrest and differentiation. Here we present evidence that the cytotaxis effect of NGF is mediated by nitric oxide (NO), a second messenger molecule with both para- and autocrine properties that can diffuse freely and act within a restricted volume^{10–14}. We show that NGF induces different forms of nitric oxide synthase (NOS) in neuronal cells, that nitric oxide (NO) acts as a cytotaxis agent in these cells, that inhibition of NOS leads to reversal of NGF-induced cytotaxis and thereby prevents full differentiation, and that capacity of a mutant cell line to differentiate can be rescued by exogenous NO. We suggest that induction of NOS is an important step in the commitment of neuronal precursors and that NOS serves as a growth arrest gene, initiating the switch to cytotaxis during differentiation.

When PC12 cells were tested for the diaphorase cytochemical reaction, no staining was observed. But after treatment with NGF, the cells gradually acquired an intense NADPH-depen-

dent blue colour after diaphorase staining, indicating that NOS accumulates in PC12 cells in response to NGF treatment (Fig. 1A). This increase in staining was specific for NGF and did not appear after addition of fetal calf serum or epidermal growth factor (Fig. 1A). The NGF-treated cells that were first to undergo initial morphological changes characteristic of the differentiated phenotype were also the first to show bright blue

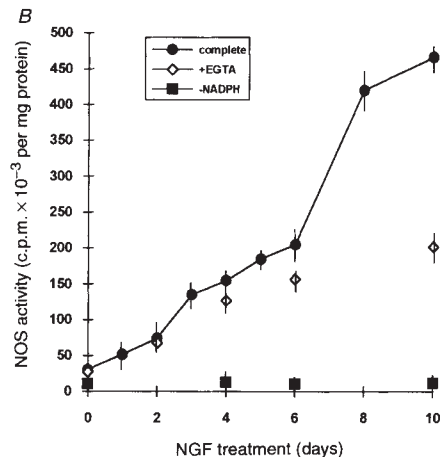


FIG. 1 NGF treatment induces NOS activity in PC12 cells. A, Diaphorase staining is induced by NGF treatment. PC12 cells were plated at low density (5×10^4 cells per ml) on collagen and poly-L-lysine-treated plates and NGF (50 ng ml⁻¹) or EGF (25 ng ml⁻¹) was added. At the indicated times cells were fixed with glutaraldehyde and tested for diaphorase staining. Scale bar, 25 μ m. B, Arginine-citrulline converting activity is induced by NGF treatment. Cell extracts were prepared after NGF treatment and NOS activity was determined by conversion of ³H-arginine to citrulline and NO in the presence or absence of EGTA and NADPH²³. NOS activity is expressed as c.p.m. of ³H-citrulline produced per mg of protein in 30 min. Error bars represent the standard error of the mean (s.e.m.). C, Immunostaining with anti-NOS antibodies. a, d, Anti-neuronal NOS monoclonal antibodies; b, e, anti-macrophage NOS monoclonal antibodies; c, f, anti-endothelial NOS monoclonal antibodies. Cells were fixed before (a–c) or after (d–f) 9 days of NGF treatment and processed for immunofluorescence using monoclonal antibodies. The exposure time for the photomicrographs of untreated cells was ~10 times longer than for NGF-treated cells; this length of exposure time was necessary to visualize the low level of immunofluorescent signal from the untreated cells. Note the differences in subcellular distribution of different NOS isoforms. Scale bar, 25 μ m.

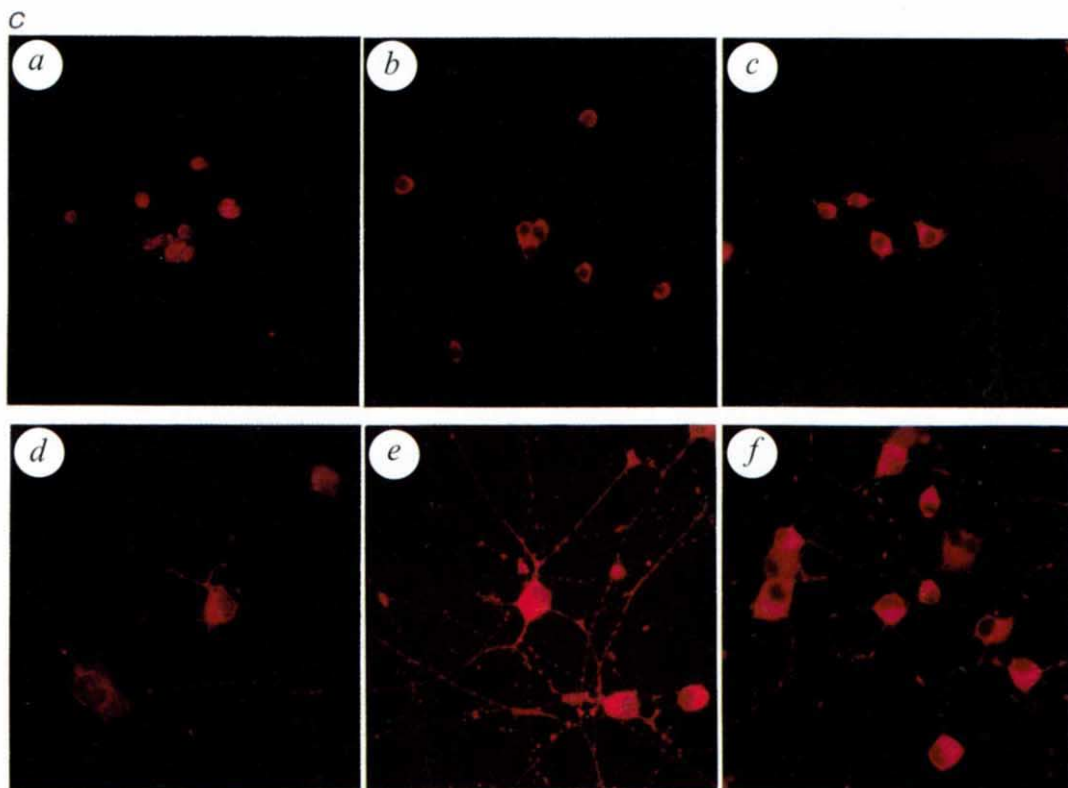
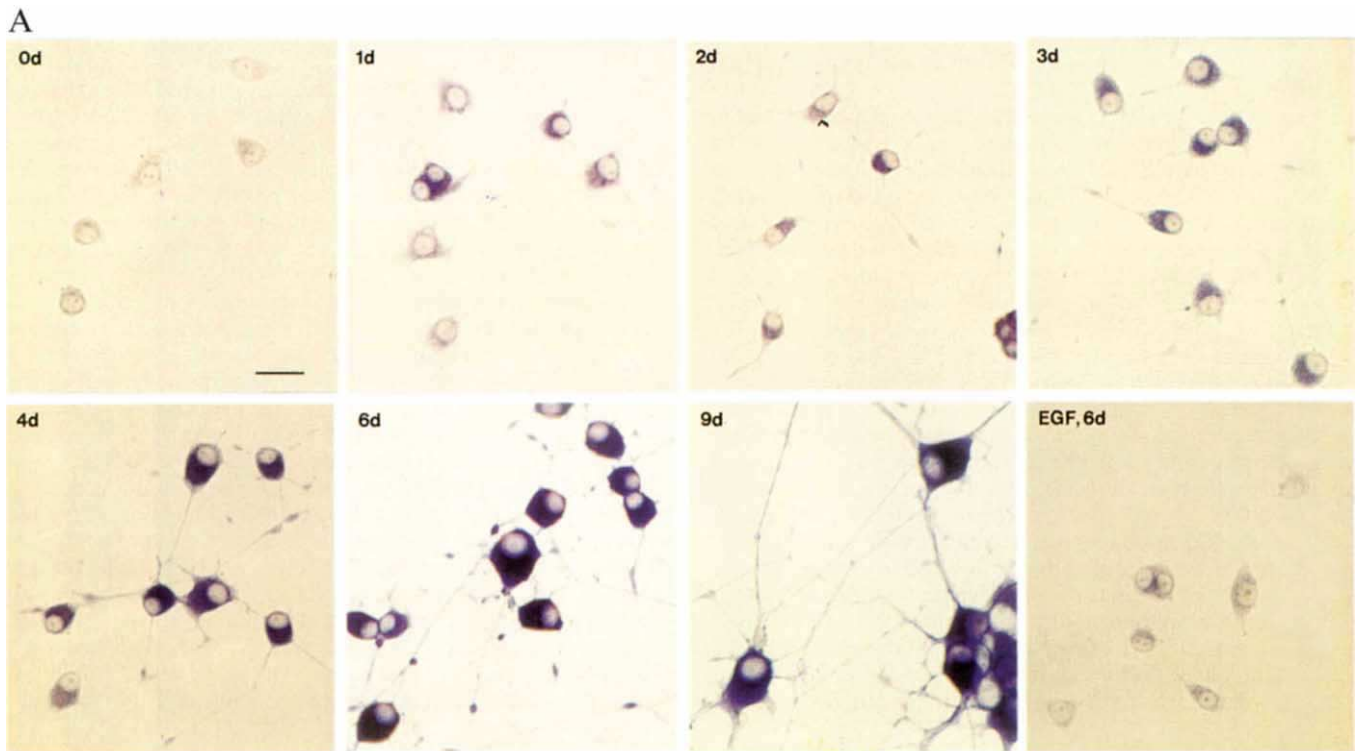
METHODS. PC12 cells were an early passage stock obtained from E. Ziff's laboratory (where they were provided by L. Greene). Cells were grown on collagen-coated plates in DMEM supplemented with 5% calf serum and 10% horse serum (HyClone). For diaphorase staining, cells were fixed with 0.2% glutaraldehyde, washed in PBS and tested for diaphorase staining with 2 mM NADPH, 0.02% tetrazolium blue and 0.3% Triton X-100 as described^{25,26}. Control experiments demonstrated that this reaction was NADPH-dependent. Diaphorase staining was not induced when cells received calf, horse, or fetal calf serum. For the ³H-arginine-citrulline conversion assay, 0.5 μ Ci of ³H-arginine was incubated with 100 μ g of cell extract together with 0.45 mM CaCl₂, 2 mM NADPH, 500 μ M arginine, 10 μ g ml⁻¹ calmodulin and 50 mM HEPES, pH 7.5, in a total volume of 50 μ l for 30 min at 37 °C as described²⁷. Citrulline was separated from arginine on Dowex AG50 columns and the radioactivity in the flow-through (citrulline-containing fraction) was determined. For determination of Ca²⁺-independent activity, the reaction was done in the presence of 3 mM EGTA. Control determinations of the enzyme activity were done in the presence of 500 μ M L-NAME, or 20 μ l of heat-treated extract, or in the absence of NADPH. For immunocytochemical analysis, cells were fixed with 0.5% paraformaldehyde for 5 min at room temperature followed by acetone for 15 min at –20 °C. The expression of various forms of NOS was visualized using anti-NOS monoclonal antibodies (Transduction Laboratories), biotinylated sheep anti-mouse antibodies (Amersham), and streptavidin-Texas red complex (Amersham). Control experiments with non-NON-specific antibodies and with omitted first antibodies gave negative results (not shown).

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staining. An *in vitro* assay showed gradual elevation of NOS activity starting at about 1 day after NGF addition, agreeing with our data on diaphorase staining (Fig. 1B).

Various forms of NOS can be discriminated by their dependence on calcium. Our data show that a large proportion of the NGF-induced NOS activity in PC12 cells (especially at the early stages of NGF action) is independent of Ca^{2+} , suggesting that different isoforms of NOS are present in NGF-treated PC12 cells. This was confirmed using immunocytochemical analysis

with specific antibodies for three different types of NOS, which revealed that differentiated cells express all three enzymes, including the calcium-dependent and independent forms (Fig. 1C). Taken together, these data indicate that elevation of NOS activity precedes further development of the differentiated phenotype, and that a substantial part of NO is produced by an inducible form of the enzyme, similar to the isoform that is induced in macrophages, hepatocytes, smooth muscle cells and other cell types after appropriate stimulation¹⁵⁻¹⁸.



NO can inhibit DNA synthesis in several systems^{19–22}. To test if NO can act as an antimitogenic agent in PC12 cells, we treated cells with increasing concentrations of three different chemical NO donors and tested the ability of the cells to incorporate ³H-thymidine. These compounds suppressed DNA synthesis in a concentration-dependent manner (albeit with different potencies), implying that NO is an active suppressor of DNA replication in PC12 cells (Fig. 2a). Both ³H-thymidine incorporation and cell proliferation were restored once NO sources were washed away, indicating that the decrease in ³H-thymidine incorporation that is seen with NO donors is caused by a decrease in DNA synthesis and cell proliferation (cytostasis) and is not due to cytotoxicity.

The changes in cell-cycle phase distribution induced by NO in PC12 cells were determined by fluorescence-activated cell sorting (FACS) analysis (Fig. 2b). At concentrations of sodium nitroprusside (SNP) up to 150 μ M, NO causes the cells to accumulate specifically in the G2 phase, whereas the proportion of cells in S phase decreases. Remarkably, within the range of SNP concentration of 30–150 μ M, the proportions of PC12 cells in G2 and S phases are similar to the levels reached after prolonged treatment with NGF (Fig. 2b and ref. 23).

After several days of NGF treatment, PC12 cell proliferation ceases and differentiation occurs^{5,6}. If NO produced by the NGF-induced NOS in PC12 cells can indeed act as an antiproliferative factor, then inhibition of the enzyme should uncouple the proliferative and cytostatic components of NGF action and prolong the proliferative phase while suppressing the cytostatic effect of NGF. To test this hypothesis, we treated PC12 cells with specific NOS inhibitors in the presence or absence of NGF and monitored both proliferation arrest and neurite outgrowth as principal manifestations of the differentiated phenotype.

NOS inhibitor *N*-nitro-*L*-arginine methyl ester (L-NAME) indeed reversed the cytostatic action of NGF and forced the cells to continue to proliferate instead of ceasing to divide after 6–8 days of NGF treatment (Fig. 3a, b). In the absence of NGF,

similar concentrations of L-NAME did not have a detectable effect on PC12 cell growth; in particular, L-NAME did not accelerate cell proliferation (Fig. 3a). Other NOS inhibitors behaved in a similar way, also permitting the cells to overcome the cytostatic activity of NGF. The inactive *D*-isomers of these compounds did not interfere with the cytostatic action of NGF, the anti-cytostatic action of NOS inhibitors was concentration-dependent, and finally, the action of L-NAME on PC12 cell division can be partially overcome by the addition of an excess of *L*- (but not *D*-) arginine (Fig. 3b).

The most visible consequence of NGF action on PC12 cells is neurite outgrowth⁵ and this was also affected by NOS inhibition. Under normal conditions, almost every cell gradually extended neurites in response to NGF but, when PC12 cells were treated with a combination of NGF and NOS inhibitor, the number of cells with neurites decreased dramatically; this effect was concentration dependent and was *L*-isomer specific (Fig. 3c, d). Thus, inhibition of NOS blocked the later stages of the terminal differentiation process; the cells did not stop dividing and they did not extend neurites.

A reciprocal test of the role of NOS induction in the differentiation cascade is to examine whether treatment with exogenous NO can influence differentiation. For these experiments we used a mutant PC12-U2 cell line⁶. These cells retain the early steps of the response to NGF, but have lost the capacity to execute the later steps. They do not stop dividing after NGF treatment and, as a consequence, they do not develop the fully differentiated phenotype; in particular, they do not send out processes. To ask if the deficit is connected to NO production, we tested if this phenotype can be overcome by the addition of NO. Figure 4a shows that although neither NGF nor NO alone had any effect on the phenotype of U2 cells, in combination these treatments restored the differentiated neuronal phenotype. The cells stopped dividing (not shown) and grew extensive branched neurites. To show the involvement of NOS in the differentiation of U2 mutant cells, the induction of NOS by NGF in these cells

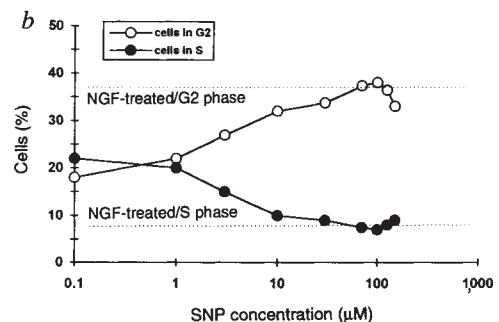
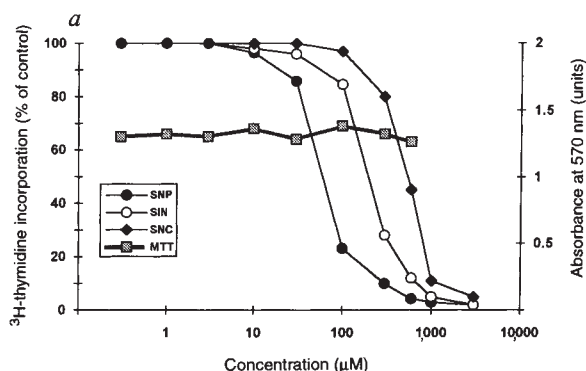
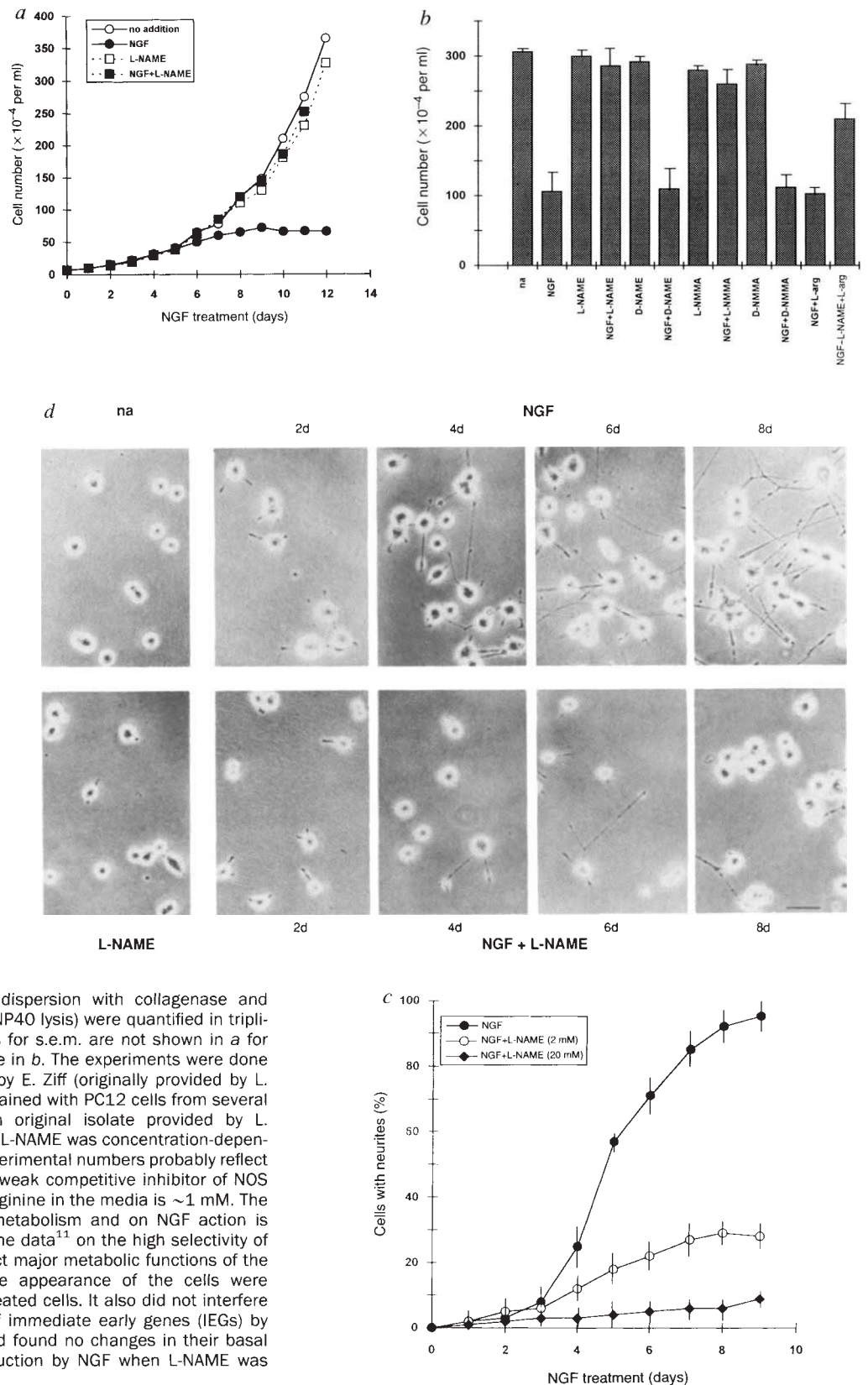


FIG. 2 Nitric oxide act as an antimitogenic cytostatic agent. **a**, Effect of NO donors on ³H-thymidine incorporation. PC12 cells were incubated with increasing concentrations of NO donors and thymidine incorporation was measured after labelling for 2 h. SNP, Sodium nitroprusside; SIN-1, 3-morpholinosydnonimine; SNC, S-nitrosocysteine; MTT, MTT tetrazolium salt conversion assay. The cells remained attached throughout the experiment and excluded trypan blue. NO-mediated inhibition of DNA synthesis overcomes induction of ³H-thymidine incorporation by serum, NGF, EGF, bFGF, serum + *N*-nitro-*L*-arginine methyl ester (L-NAME), and NGF + L-NAME (data not shown). **b**, Effect of SNP on cell-cycle phase distribution measured by FACS analysis. Changes in cell-cycle phase distribution after exposure to increasing concentrations of the NO donor sodium nitroprusside (SNP) are presented as the proportion of cells in G2 and S phases. For comparison, the fractions of cells in G2 and S phases after 9 days of NGF treatment are shown by the dotted lines.

METHODS. For thymidine incorporation experiments, cells were grown on medium with 1% serum for three days, transferred to medium with

15% serum, incubated with various concentrations of NO donors for 16 h as indicated (duplicates for each concentration) and labelled with tritiated thymidine for 2 h. Cells were collected and the incorporated radioactivity was determined in duplicate for each sample. Control experiments have shown that NO donors affect the incorporation of radioactive thymidine into DNA rather than interfere with the thymidine uptake. For the cytotoxicity analysis the cellular conversion of the MTT tetrazolium salt into formazan after incubation with increasing concentrations of SNP was determined using a reagent kit from 'Promega'. The assay was done as recommended by the manufacturer and data are presented as absorbance of formazan at 570 nm. For FACS analysis, cells were incubated with increasing concentrations of SNP for 16 h; nuclei were prepared in the hypotonic buffer with 0.2% NP40, fixed with ethanol, treated with RNase, incubated with 50 μ g ml⁻¹ propidium diiodide, and analysed for DNA content distribution using Coulter EPICS Elite flow cytometer. Similar data were obtained when cells were analysed by combining propidium diiodide staining with an immunofluorescent antibody to BrdU after BrdU incorporation (data not shown).

FIG. 3 Inhibition of nitric oxide synthase prevents cytostatic action of NGF and neurite outgrowth. **a**, Growth of NGF-treated PC12 cells with and without L-NAME. PC12 cells were plated at low density and were treated with NGF (50 ng ml⁻¹), L-NAME (1 mM), a combination of both, or received no additions. **b**, Effect of NOS inhibitors on cell proliferation. PC12 cells were treated with NGF alone or in combination with L-NAME (1 mM), D-NAME (5 mM), *N*-monomethyl-L-arginine (L-NMMA) (3 mM) or D-NMMA (3 mM), and L-arginine (8 mM). At day 9, the cells were counted in triplicate as described. Error bars represent s.e.m. **c**, Neurite outgrowth in NGF-treated PC12 cells with and without L-NAME. Cells received NGF (50 ng ml⁻¹) with or without 2 mM or 20 mM L-NAME. Cells with neurites were counted as a percentage of total cells in five independent areas of the plate. Only the cells with neurites longer than two cell diameters were scored as positive. Error bars represent s.e.m. At low concentrations of inhibitor (0.5–5 mM), about 25% of cells still extended neurites, although these neurites were fewer in number (per cell), shorter and less branched than those observed in cells that were treated with NGF without L-NAME. At high L-NAME concentrations (8–20 mM), NGF-induced neurite outgrowth was almost completely suppressed. **d**, Phenotype of PC12 cells treated with NGF, L-NAME and combinations. PC12 cells were plated at low density on collagen-treated plates and NGF (50 ng ml⁻¹) and L-NAME (5 mM) were added. Photomicrographs were taken at the days indicated. Scale bar, 50 μ m.



METHODS. Cells (after proteolytic dispersion with collagenase and papain) and nuclei (after hypotonic NP40 lysis) were quantified in triplicate (three plates per point). Values for s.e.m. are not shown in **a** for clarity, but they were similar to those in **b**. The experiments were done with a PC12 cells isolate provided by E. Ziff (originally provided by L. Greene), but similar results were obtained with PC12 cells from several independent sources, including an original isolate provided by L. Greene. The anti-cytostatic action of L-NAME was concentration-dependent, starting at ~ 100 μ M; these experimental numbers probably reflect the fact that L-NAME is a relatively weak competitive inhibitor of NOS and that the concentration of free arginine in the media is ~ 1 mM. The effect of L-NAME on general cell metabolism and on NGF action is rather specific, in accordance with the data¹¹ on the high selectivity of NOS inhibitors. L-NAME did not affect major metabolic functions of the cell, so that growth rates and the appearance of the cells were unchanged in comparison with untreated cells. It also did not interfere with the rapid induction of a set of immediate early genes (IEGs) by NGF. We tested a panel of IEGs and found no changes in their basal levels of expression or in their induction by NGF when L-NAME was added (data not shown).

was examined by immunofluorescence. Figure 4b shows that inducible NOS is expressed at much lower levels in U2 cells than in the wild-type PC12 cells after 4 days of NGF treatment (note the difference in exposures). At this same time, the levels of the neuronal and endothelial forms of NOS in U2 cells are indistinguishable from those in PC12 cells (data not shown). Taken

together, these data demonstrate that the NGF-treated U2 cells are deficient in inducible NOS and that this defect can be rescued by complementing NGF action with NO.

Experiments in which exogenous NO was added to the mutant U2 cells complement the experiments with wild-type PC12 cells in which NO production was blocked. Together they indicate a

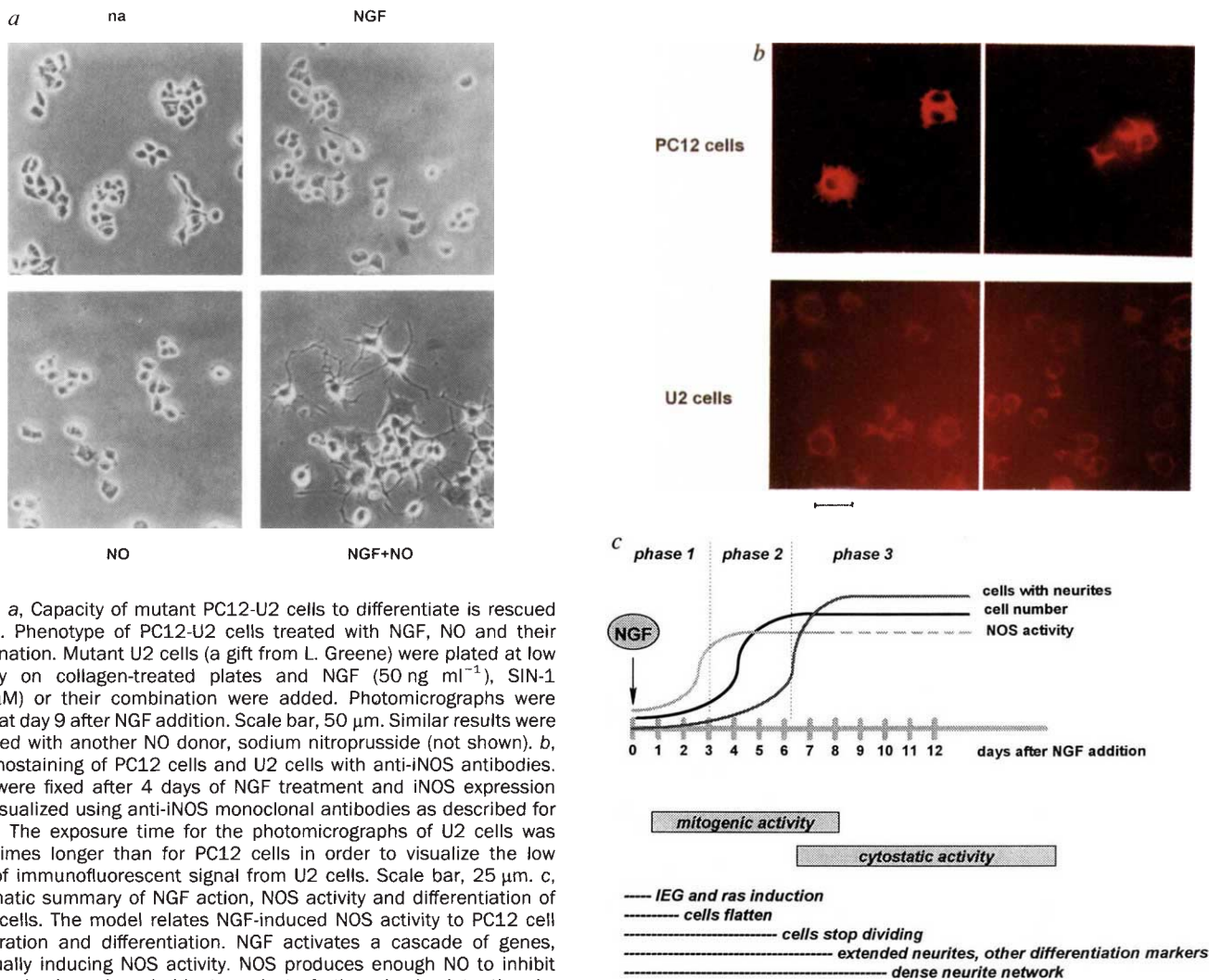


FIG. 4 *a*, Capacity of mutant PC12-U2 cells to differentiate is rescued by NO. Phenotype of PC12-U2 cells treated with NGF, NO and their combination. Mutant U2 cells (a gift from L. Greene) were plated at low density on collagen-treated plates and NGF (50 ng ml^{-1}), SIN-1 ($300 \mu\text{M}$) or their combination were added. Photomicrographs were taken at day 9 after NGF addition. Scale bar, $50 \mu\text{m}$. Similar results were obtained with another NO donor, sodium nitroprusside (not shown). *b*, Immunostaining of PC12 cells and U2 cells with anti-iNOS antibodies. Cells were fixed after 4 days of NGF treatment and iNOS expression was visualized using anti-iNOS monoclonal antibodies as described for Fig. 1. The exposure time for the photomicrographs of U2 cells was ~ 10 times longer than for PC12 cells in order to visualize the low level of immunofluorescent signal from U2 cells. Scale bar, $25 \mu\text{m}$. *c*, Schematic summary of NGF action, NOS activity and differentiation of PC12 cells. The model relates NGF-induced NOS activity to PC12 cell proliferation and differentiation. NGF activates a cascade of genes, eventually inducing NOS activity. NOS produces enough NO to inhibit DNA synthesis and, probably, to activate further checkpoints, thereby blocking the cell cycle progression, completing the proliferative phase and switching the cells to the cytostatic phase. Finally, after growth arrest, the cell starts to implement those differentiation traits that can only occur after cell division has ceased (such as neurite outgrowth). Various forms of NOS, induced with different kinetics, might perform different physiological functions during PC12 cell differentiation.

causative role for NO action in NGF-induced growth arrest and differentiation in PC12 cells.

The results of this study suggest a model for NGF action in PC12 cells in which at least three stages can be outlined (Fig. 4c). In the first, proliferative stage NGF activates a cascade of genes, eventually leading to the induction of the NOS gene. In the second stage, the accumulated NOS enzyme produces enough NO to inhibit DNA synthesis and, probably, to 'alert' further checkpoints, thereby blocking further progression of the cell cycle, completing the proliferative phase of NGF action, and switching the cells to the cytostatic phase; probably, NO also directly induces some of the later differentiation markers by promoting gene activity²⁴. Finally, as soon as the cell perceives and processes the cytostatic signal, it starts to implement the remaining programme of differentiation traits (such as neurite outgrowth), which can only occur after cell division has ceased.

Is a similar NOS-mediated mechanism used by other systems (including, but not limited to, the nervous system) to maintain mitogenic quiescence? NOS-dependent cytostasis could explain the action of other growth factors during terminal differentiation in which the initial mitogenic response is replaced by a cytostatic phase. It might also help to explain the regulated mitogenesis in

Inducible NOS might be primarily responsible for a slow accumulation of cytostatic activity (independent of temporary calcium stimulation), whereas the neuronal (and, perhaps, endothelial) form of NOS might be primarily important for the later stages of development in fully differentiated cells, where transient modulations of calcium level activate NOS to induce secretion of neurotransmitters²⁸.

tissues expressing NOS (endothelial cell regeneration, liver injury, atherosclerotic smooth muscle cell lesions, wound healing and so on) where initial rapid proliferation has to be followed by controlled cessation in order not to provoke excessive growth. Given the unconventional properties of NO it is possible that NO diffuses from the cell that produces it to promote cessation of growth in adjacent cells. If NO molecules, produced by a group of adjacent cells, act additively within a limited volume, this could contribute to the synchronization of development of a domain of precursor cells. Finally, because NO is also involved in neuronal plasticity, induction of NOS could provide a link between the activity of a cell and the developmental fate of it and its nearest neighbours. □

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A calcium-channel homologue required for adaptation to dopamine and serotonin in *Caenorhabditis elegans*

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PROCESSING and storage of information by the nervous system requires the ability to modulate the response of excitable cells to neurotransmitter. A simple process of this type, known as adaptation or desensitization, occurs when prolonged stimulation triggers processes that attenuate the response to neurotransmitter. Here we report that the *Caenorhabditis elegans* gene *unc-2* is required for adaptation to two neurotransmitters, dopamine and serotonin. A loss-of-function mutation in *unc-2* resulted in failure to adapt either to paralysis by dopamine or to stimulation of egg laying by serotonin. In addition, *unc-2* mutants displayed behaviours similar to those induced by serotonin treatment. We found that *unc-2* encodes a homologue of a voltage-sensitive calcium-channel α -1 subunit. Expression of *unc-2* occurs in two types of neurons implicated in the control of egg laying, a behaviour regulated by serotonin. *Unc-2* appears to be required in modulatory neurons to downregulate the response of the egg-laying muscles to serotonin. We propose that adaptation to serotonin occurs through activation of an *Unc-2*-dependent calcium influx, which modulates the post-synaptic response to serotonin, perhaps by inhibiting the release of a potentiating neuropeptide.

To identify molecules involved in adaptation in the *C. elegans* nervous system, we searched for mutants that failed to adapt to

neuroactive substances. We focused primarily on two biogenic amines, dopamine and serotonin. Both compounds are found in *C. elegans* neurons^{1,2}, and both have striking effects on nematode behaviour. Exogenous serotonin has several behavioural effects: stimulation of egg laying, inhibition of locomotion, stimulation of feeding, and activation of a specific step in the male mating program^{2–4}. We found that dopamine treatment had at least two effects on *C. elegans* behaviour: inhibition of movement, and inhibition of egg laying (Table 1). Thus dopamine and serotonin had opposing effects on egg laying and similar effects on movement, although serotonin, unlike dopamine, also caused animals to move in a twisting, 'kinking' manner. Sets of neurons expressing either serotonin or dopamine have been identified⁵, including the serotonergic hermaphrodite-specific neurons (HSNs)⁵, required for egg laying, and the CP neurons, which also contain serotonin and are involved in male mating⁴.

To determine whether *C. elegans* could adapt to these neurotransmitters, we tested the response to dopamine and serotonin after prolonged exposure. Treatment with 3 mg ml⁻¹ (16 mM) dopamine initially inhibited egg laying and locomotion in wild-type animals. However, animals treated with dopamine in this manner for 4 hours or more recovered the ability to move and lay eggs normally. These pretreated animals became resistant to inhibition of both egg laying and locomotion by up to 6 mg ml⁻¹ dopamine, indicating that they had adapted to dopamine (Table 1; Fig. 1a, c). Moreover, when these adapted animals were transferred to a solution that did not contain dopamine, they laid eggs at an abnormally high rate, suggesting that they had become dependent on exogenous dopamine for the control of egg laying (Table 1). Adapted animals regained sensitivity to dopamine over the course of approximately 4 hours (Fig. 1b). Long-term exposure to serotonin also led to adaptation. Serotonin (3 mg ml⁻¹, 7.7 mM) initially stimulated egg laying; however, animals exposed to serotonin overnight accumulated unlayed eggs, and were unable to lay eggs in response to a fresh dose of serotonin (Table 1, Fig. 2d).

TABLE 1 Adaptation to dopamine and serotonin

Experiment	Strain genotype	Pretreatment	Test conditions	Eggs laid (eggs per worm per hour)	Percentage active
1	wild-type	no drug	no drug	2.5 [$\pm$ 1.1]	100
	wild-type	no drug	6 mg ml ⁻¹ dopamine	0.5 [$\pm$ 0.1]	0
	wild-type	3 mg ml ⁻¹ dopamine	6 mg ml ⁻¹ dopamine	2.5 [$\pm$ 1.7]	100
2	<i>egl-1(n987)</i>	no drug	no drug	0.6 [$\pm$ 0.2]	
	<i>egl-1(n987)</i>	no drug	3 mg ml ⁻¹ serotonin	45.2 [$\pm$ 6.8]	
	<i>egl-1(n987)</i>	3 mg ml ⁻¹ serotonin	3 mg ml ⁻¹ serotonin	0.8 [$\pm$ 0.8]	
3	wild-type	no drug	no drug	3.6 [$\pm$ 2.8]	
	wild-type	3 mg ml ⁻¹ dopamine	no drug	9.8 [$\pm$ 2.8]	

Effects of adaptation on egg laying and locomotion. Wild-type or *egl-1* adult hermaphrodites were grown overnight on 1.5% agar plates spread with *Escherichia coli* strain OP50, and with dopamine hydrochloride or serotonin creatinine sulphate added at the indicated concentrations (2 mM acetic acid was added to dopamine plates to stabilize the dopamine). To eliminate effects of endogenous serotonin, *egl-1* mutants, which lack HSN neurons, were used for the serotonin experiment. After overnight incubation in the presence or absence of drug, 10 animals were transferred to freshly poured test plates containing dopamine or serotonin as indicated, and were allowed to lay eggs at room temperature. Eggs were counted after 45 minutes for experiment 1, 1 hour for experiment 2, and 30 minutes for experiment 3. These data represent the mean rate of egg laying in 4 or more independent experiments (experiment 3 involved 11 independent trials). The sample standard deviation of these data is indicated in brackets.

We identified adaptation-defective mutants by looking for animals that remained sensitive to 3 mg ml^{-1} dopamine after prolonged treatment. One of the mutants identified in such a screen, *mu74*, was found to be a recessive allele of the previously identified gene *unc-2* (refs 6, 7). The acute response of *unc-2(mu74)* animals to dopamine was essentially wild type (Fig. 2a), yet they were severely defective in desensitization; nearly all *unc-2(mu74)* animals treated with 3 mg ml^{-1} dopamine failed to recover the ability to move after 14 hours (Fig. 2b). Thus the *unc-2(mu74)* mutation appeared to disrupt adaptation specifically. We tested the desensitization phenotypes of four other *unc-2* alleles: three alleles, *e55*, *e97* and *e129*, also had a strong dopamine adaptation-defective phenotype; a fourth allele, *e2379*, had only a subtle defect in dopamine adaptation (Fig. 2b). We found that *unc-2* mutants also exhibited several distinctive behavioural abnormalities, including constitutive egg laying (Fig. 2c) and slow, kinking movement. This behavioural phenotype mimicked the effect of serotonin treatment on wild-type animals, raising the possibility that *unc-2* mutants might also be unable to adapt to endogenous serotonin. The *unc-2* animals continued to lay eggs in response to serotonin even after overnight treatment (Fig. 2d); thus the *unc-2* gene appeared to be required for adaptation to serotonin as well as dopamine.

To understand the biochemical function of the *unc-2* gene product, we cloned the *unc-2* gene (Fig. 3). We found that *unc-2* encodes a predicted polypeptide product with high sequence

similarity to the α -1 subunit of voltage-sensitive calcium channels⁸⁻¹¹. Although this protein did not appear to be the precise counterpart of a particular mammalian channel subtype, its sequence was more similar to neuronal α -1 subunits of the A, B and E (non-L-type) classes than to channel subunits of the C, D, and L_{skel} (L-type) classes. The *unc-2(mu74)* mutation deleted a predicted transmembrane α -helix in the last of four membrane-spanning domains in the putative ion pore¹²; thus the mutant protein should have decreased channel function (Fig. 3). Interestingly, a mutation in the gene *unc-36*, which encodes a putative voltage-gated calcium-channel α -2 subunit (L. Lobel and H. R. Horvitz, personal communication), produces essentially the same phenotype as mutations in *unc-2*; we have found that *unc-36* mutants are also defective in dopamine adaptation (W.R.S. and C.J.K., results unpublished). Thus, *unc-2* and *unc-36* may encode subunits of a voltage-gated calcium channel that participates in adaptation to dopamine and serotonin.

How might this channel act within the *C. elegans* nervous system to mediate behavioural plasticity? We focused initially on serotonin adaptation and egg-laying behaviour. Anatomical and pharmacological data implicate two classes of neurons, one of them serotonergic, in the control of egg laying. The two HSN motor neurons, which synapse onto the vulval muscle, are thought to release serotonin to activate muscle contraction^{13,14}. Six additional, ventral type C (VC), neurons receive input from HSN, and provide synaptic output to the vulval muscles¹³; both

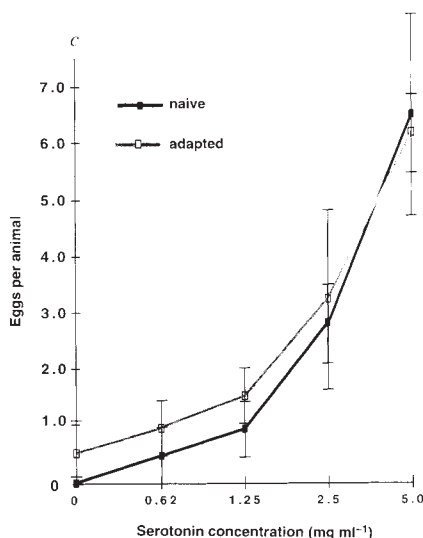
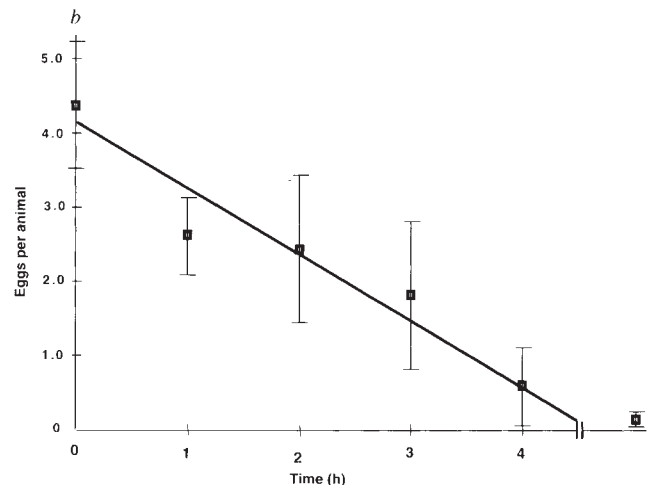
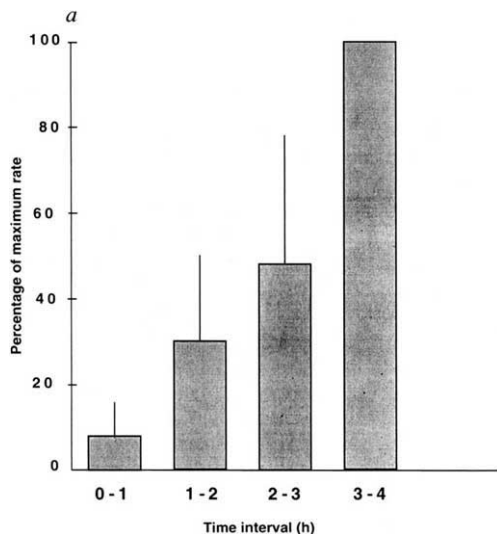


FIG. 1. Adaptation to dopamine and serotonin. **a**, Time course of dopamine adaptation. Animals were placed on 1.5% agar plates containing 3 mg ml^{-1} dopamine at 20°C ; eggs were counted at 1-h intervals until the maximum rate of egg laying (mean = 4.0) was reached. Data represent the mean percentage of the maximum rate for the indicated time interval. Mean and sample standard deviations were calculated from 4 populations of 10 animals each. **b**, Time course of dopamine resensitization. Animals were adapted overnight on 1.5% agar containing 3 mg ml^{-1} dopamine as above. Adapted animals were then transferred to 1.5% agar containing 3 mg ml^{-1} dopamine as above. Adapted animals were then transferred to 1.5% agar plates lacking dopamine. At the indicated time points, groups of 10 animals were moved back to plates containing dopamine (4.5 mg ml^{-1}) and allowed to lay eggs. Each point and error bar indicates the mean and sample standard deviation, respectively, of 4 sets of 10 animals. **c**, Serotonin response of dopamine-adapted animals. To determine whether dopamine adaptation affected the response to serotonin, animals were grown overnight on 1.5% agar plates in the presence (adapted) or absence (control) of 3 mg ml^{-1} dopamine as above. The ability of serotonin to stimulate egg laying was assayed in liquid culture as described¹⁴. Points and error bars indicate the mean and sample standard deviation of 3 sets of 10 animals. In all experiments, low-calcium agar was used, as calcium inhibits dopamine response in our assay.

the VCs and the HSNs produce a FMRFamide-like neuropeptide that can facilitate the ability of serotonin to stimulate egg laying¹⁵. *In situ* hybridization experiments indicated that *unc-2* message is expressed in both the HSN and VC neurons; in contrast, *unc-2* expression was not detected in the egg-laying muscles (Fig. 4a). Functional evidence that Unc-2 protein acts in neurons was obtained through analysis of mosaic animals¹⁶ that were composed of both wild-type and *unc-2* mutant cells. In these mosaic animals, constitutive egg-laying behaviour correlated with lack of a functional *unc-2* gene in neuronal lineages, suggesting that the *unc-2* gene product is required in neurons to regulate egg laying (Fig. 4b).

In principle, the constitutive egg-laying behaviour of *unc-2* mutants could result from elevated release of serotonin from the

HSNs, or from an abnormally sensitive response to serotonin by the egg-laying muscles. To investigate these possibilities, we eliminated the HSN neurons (the endogenous source of serotonin) using a mutation in the gene *egl-1* (ref. 14), which causes them to undergo cell death. If the constitutive egg-laying phenotype of *unc-2* mutants results exclusively from inappropriate serotonin release by the HSNs, then once the HSNs have been eliminated, *unc-2* and wild-type animals should exhibit the same response to serotonin. Alternatively, if the *unc-2* mutation causes an increased response to serotonin, the *unc-2* mutant lacking the HSNs should be serotonin hypersensitive. In fact, this was the case; the *egl-1; unc-2* double mutant failed to lay eggs in the absence of serotonin, but was sensitive to nearly tenfold lower concentrations of serotonin than either

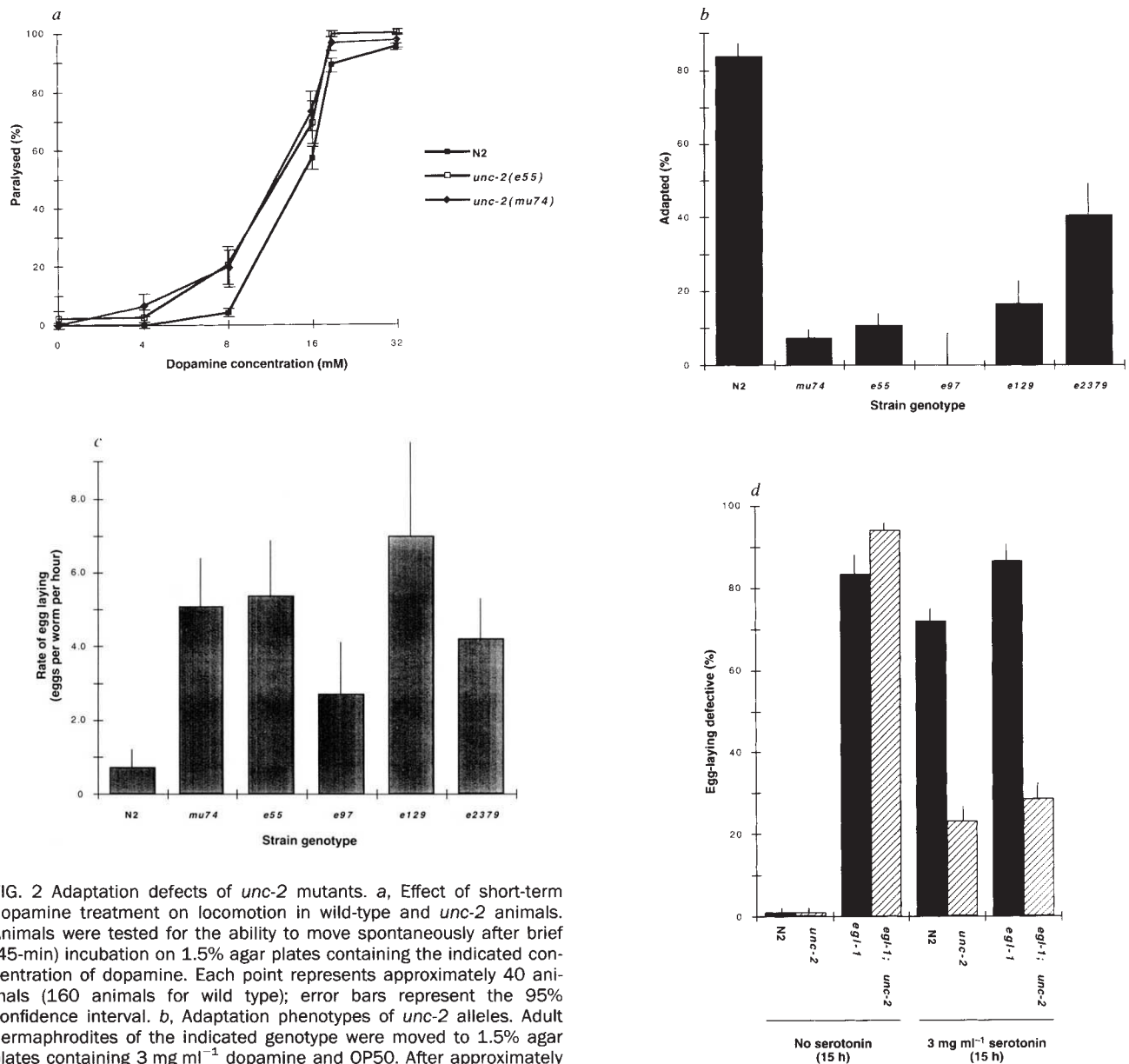
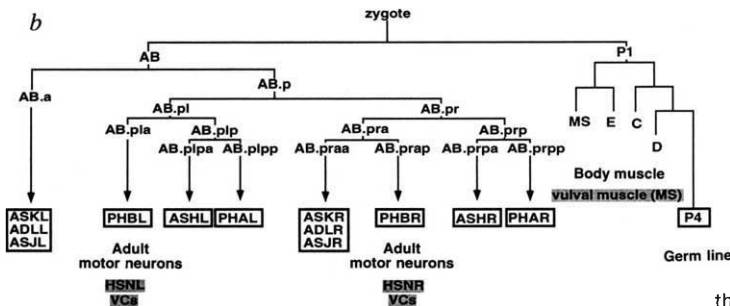
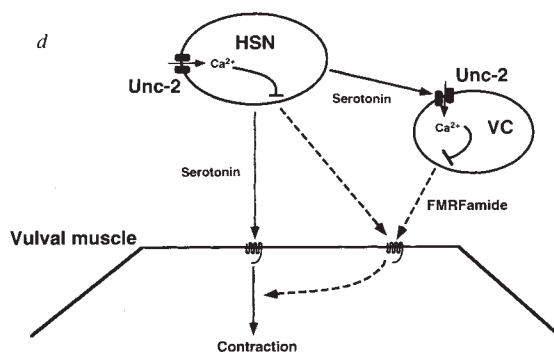
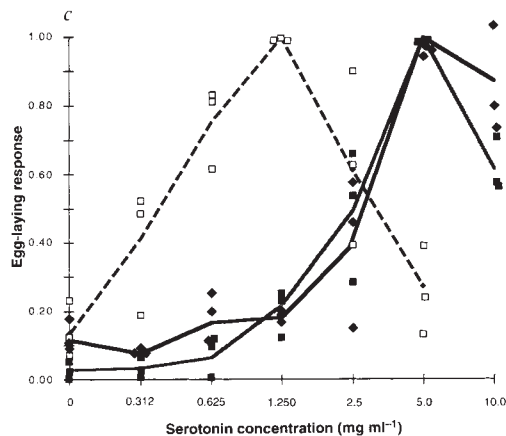
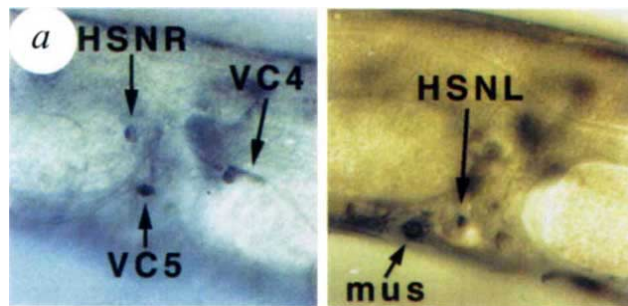


FIG. 2 Adaptation defects of *unc-2* mutants. **a**, Effect of short-term dopamine treatment on locomotion in wild-type and *unc-2* animals. Animals were tested for the ability to move spontaneously after brief (45-min) incubation on 1.5% agar plates containing the indicated concentration of dopamine. Each point represents approximately 40 animals (160 animals for wild type); error bars represent the 95% confidence interval. **b**, Adaptation phenotypes of *unc-2* alleles. Adult hermaphrodites of the indicated genotype were moved to 1.5% agar plates containing 3 mg ml⁻¹ dopamine and OP50. After approximately 15 h the percentage of animals that had adapted to dopamine was determined by counting the number of active (adapted) and paralysed (non-adapted) animals. Error bars represent the 95% confidence interval. The following numbers of animals were tested: N2, 105; *mu74*, 138; *e55*, 75; *e97*, 31; *e129*, 36; *e2379*, 32. **c**, Constitutive egg laying in *unc-2* mutants. Egg-laying rates for wild-type and mutant animals were determined during a 1-h incubation in liquid culture as described¹⁴. Bars represent the overall mean rate from 5 experiments of 10 animals each; error bars indicate the sample standard deviation

of the 5 individual mean rates. **d**, Chronic serotonin response in *unc-2*(+) and *unc-2*(*e55*) animals. Young adult hermaphrodites were incubated overnight on 3 mg ml⁻¹ serotonin as in Table 1. After approximately 15 h, animals that had accumulated unlayed eggs were scored as egg-laying defective. Each bar represents the percentage of egg-laying defective animals among at least 65 animals. Error bars indicate 95% confidence intervals for the difference between the *unc-2* mutant and *unc-2*(+) strain under each condition.

IIS1										IIS2										IIS3									
Unc-2	IQIRIMVKTQ	IFWNSVITLV	FLNCCVASE	KYQGPQMTD	FLKYAFVFL	GIFVVDMLK	LFAMGRTYF	ASKFNRFDCV	VIVGSAEVI	NAEYVGG.SF	99																		
rbB-I (474)	FL--R--A	S--V-LCV	A--L--MV	-N--RL-T	A-YF----	-L-LT--S-	MYGL-P-S-	R-S-C--FG	-----IF--V	--AIKF-T--	573																		
rbA-I (480)	FY--R----	A--T--LS-	A--LML-IV	-N--E-LSD	-Y--I--	-L-MS--FI	MYGL-T-P-	H-S-C--G	-I--IF--	--VIKF-T--	579																		
rbE-II (418)	-S--H--S-	V--I--LSV	A--A--IV	-HN--L-H	L-Y--L-	-L-LL--S-	MYQ--P-L-	H-S-C--FG	-T--IF--V	--IFRP-T--	517																		
rbC-I (515)	RKC-AA--SN	V--L--FL-	---LTI-	-N--H-L-E	VQDT-NKAL	AL-TA----	MYSL-LQA-	V-L-----	F	IVC-GIL-T-	LV-TKMSPL	614																	
IIS4										IIS5																			
Unc-2	GISVMRALRL	LRIFKLTSM	VSLRNLVRL	MNSRSIISL	LFLFLFLILI	FALLGMQLFG	GRPNFPT.MH	PYTHFDTPV	ALITVFQILT	GEDWNEVMYL	198																		
rbB-I	---L---	---V-K-	N-----V-	L--K----	-----VV	-----	-Q--QD.ET	T-N-----	-IL-----	---A---K	672																		
rbA-I	---L---	---V-K-	AS-----V-	L--K----	-----VV	-----	-Q--DE.GT	P-N-----	-IM-----	---H---D	678																		
rbE-II (418)	---L---	---I-K-	A-----V-	S--K----	-----VV	-----	---ND.GT	-SAN-----	-IM-----	---N---D	616																		
rbC-I	---L-CV--	---I-R-	N--S--A-	L--V--A-	-L-----I-	-S-----	-K--DEMGT	RRST--N-Q	S-L-----	---S---D	957																		
IIS6																													
Unc-2	AIESQGGIYS	GGWFSIYFI	VLVLFQNYTL	LVFLAIAVD	MLANAQLTA	AREADEKANE	IERESELDE	QYQGDHCTI	DMEGKTAGEM	CAVARAMDDL	798																		
rbB-I	G-----VSK	-M.FS-F-	---T-----	-----K	D--EM-E-AN						231																		
rbA-I	E-K-----VQG	-M.VF-	---T-----	-----K	D-QEE-E-AN						677																		
rbE-II	G-R-----VS	-M.SA-	---T-----	-----K	D-QEE-E-FH						735																		
rbC-I	G-MAY--PSF	PGMLVC-	I-FIC--I-	-----	-D-S--S	AQKEE-EEK-					774																		
IIIS1										IIIS2																			
Unc-2	DEECHEESP	FGGPKPMVFP	SSMFLSPTN	PFVLIHSIV	CTKTFEGVM	TVICLSSVSL	AAEDPVDERN	PRNKVLQMD	YCFITGLACE	MLLKLIQGI	398																		
rbB-I (1108)	-A-ADDVL	RR--R-I--	---C---	LL-RFC-Y-	TMR--VIL	V--A--IA	-----RTDS	F--NA-K--	-I--FTF-	-VI-M--L-L	1205																		
rbA-I (1155)	-E--ADPG	ED-----P-	I--T--	-L-R-C-Y-L	NLR--CIL	M--AM--IA	-----OPNA	-N--R-F-	-V--FTF-	-VI-M--L-L	1252																		
rbE-II (1066)	KKQKK-KR	ET--A--H	---IF-T--	-I-KAC-Y-	NLR--CIL	L-AA--IA	-----LTNS	E--R--F-	-V--FTF-	-VI-M--L-L	1162																		
rbC-I (860)	PLS-LHLK	EKAV-MPEA	-AF-IF--H-	R-LQC-R--	MDTI-TNLI	FF-L--IS-	-----QHTS	F--HI-GNA-	-V--SIFTL-	II--MTAY-A	957																		
IIIS3										IIIS4										IIIS5									
Unc-2	LLHPSGICRD	FWNILDGIVV	TCALFAGFA	G.TEBSAGKN	LNTKSLRVL	RVLRLPLTKI	RIPKLKAVFD	CVVNSLKNVF	NILIVYFLQ	FIFAVIAVQ	497																		
rbB-I	---A-F--	L-----F-	SG--A-S	SPMG-K-D	I-----	---L-----	-----L	-----M--M	-----	-----	1305																		
rbA-I	V--Q-A-F--	L-----F-	SG--V-A-T	NSK-KDINT	IKSLRV-	-P-KTI-RLP	KLKAV--	-----	-----M-FM	-----V--	1349																		
rbE-II	I-QD--F--	L-----FV-	VG--V-AL-	NALGTNR-RD	IK--	---L-----	-----T	-----K--M	-----	-----	1262																		
rbC-I	F--K--F--N	YF--LL-	SVS-IS--I-	QSSA	I-VV-I--	-----RA-N	-AKG--HVQ	-FVAIRTIG	-V--TT-L-	-M--CIG--	1050																		
IIIS6																													
Unc-2	FGHGFCTD	KNRKANTCH	GQFFVNDQN	DPFVEQRE	WRLPFNYDN	TINAMTLFV	VTTQEGWPI	RQNSMDTTFE	DQSPSPFRV	EVALFYVMFF	596																		
rbB-I	-K--Y--	ESKELERD-R	-YLD-EKEE	...VBAQP-Q	-KKYD-H--	VLM-L--T	-S-----MV	LKH-V-A-Y	E-----G-M	-LSI--VY-	1402																		
rbA-I	-K--Y--	ESKELERD-R	-KYLL-EKNE	...VKARD-	-KKYD-H--	VLM-L--T	-S-----QV	LKH-V-A-Y	E-----GY-M	-MSI--VY-	1446																		
rbE-II	-K--Y--	SSKDTKEE-I	-HYVDHEKNK	...ME-KG-	-KRHE-H--	IIV-L--T	-S-----QV	LQH-V-V-E	-R--R--SN-M	-MSI--VY-	1359																		
rbC-I	-K--LYT-S	SSKQTEAE-K	-HYIT-KDGE	V-H-IIQP-S	-ENSK-DF--	VLAAMMALFT	VSTFEGWPEL	LYR-I-SHT	-K--IYNY-V	-ISI-FIYI	1150																		
IVS1																													
Unc-2	IVFVFVFNK	FVALIITFQ	EQGEALSEG	DLKQKQCI	DFGLNARPS	LFMPEDKNT	KYRIWRLVTS	PPFFYFIMTM	ICCNLTILMM	KYNNPLFYE	696																		
rbB-I	V-----	-----	---DKVM-C	S-E--ERA-	-AIS-K-LT	RY--QN-Q-F	Q-KT-TF-V	-----A-	-AL-VV--	-F-DA-YE--	1502																		
rbA-I	V-----	-----	---DKME-Y	S-E--ERA-	-AIS-K-LT	RH--QN-Q-F	Q-M-QF-V	-----T-A-	-AL-IV--	-F-GASVA--	1546																		
rbE-II	V-----	-----	---DKME-C	S-E--ERA-	-AIS-K-LT	RY--QNRHTF	Q-V-HP-V	-S--T-A-	-AL-VV--	-SA-WT--	1459																		
rbC-I	-IIA--MM--	---QFV-V--	---Q-YKNC	E-----R-V	EYA-K--LR	RYI-KNGH--	Q-KV-YV-N	TY--LMFVL	-LL--IC-A-	QH-GQSLFK	1248																		
IVS2										IVS3										IVS4									
Unc-2	EILRL	FGHGFCTD	KNRKANTCH	GQFFVNDQN	DPFVEQRE	WRLPFNYDN	TINAMTLFV	VTTQEGWPI	RQNSMDTTFE	DQSPSPFRV	EVALFYVMFF	781																	
rbB-I	LM-KCL-IVF	-SM-SL-C-	---I--L-	---A--V-	---L--I-	-----I-	-----I-	-----I-	-----I-	-----I-	1587																		
rbA-I	NA--V-IVF	-SL-SL-CV-	---VM--IL-	---A--I-	---L--I-	-----I-	-----I-	-----I-	-----I-	-----I-	1631																		
rbE-II	LA-KYL-I-F	-M-SL-CV-	---VI--FL-	---T--I-	---I--I-	---TEI-	IL-DSKLV-	-----	NTSGFMS-	K-----K	1547																		
rbC-I	IAMNLI-MLF	-GL--M--	---KPKG-	-S-P-V-	LI-I--I-V	ILS-TNPAKH	TQCSPSMSAE	ENSRI-IT-F	-----VM--V-	-SR-EG--T	1348																		
IVS5																													
Unc-2	LLWTFVQSF	ALPFCILLIG	MLFFIYAIVO	MQVFGNIWLN	AATE.....	..INRHNFQ	SFFNAVILLF	RCATGEGWQD	IMMAVQGGK	CARA.GSAE	872																		
rbB-I	-----A	-----I	-----A	-D-G-S-	-----	FQ-TE--R	T-Q-LM--	-S--A-HE	-LSCLGNRA	-DPH-AN-S-	1677																		
rbA-I	-----A	-----I	-----A	-D-G-S-	-----	FQ-TE--R	T-Q-LM--	-S--A-HE	-LSCLGNRA	-DPH-AN-S-	1731																		
rbE-II	-----A	-----I	-----A	-D-G-S-	-----	FQ-TE--R	T-Q-LM--	-S--A-HE	-LSCLGNRA	-DPH-AN-S-	1638																		
rbC-I	---IK-Q	---A-V	---VI--	---K-A-	DT-----	..N--	T-Q-L-	-----A-	-L-CMP-K	-PE-SRPSN	1439																		
IVS6																													
Unc-2	NFRGQTCGS	NVSYAYTSP	VFLSSFLMLN	LFVAIVMDNF	DYLRDSSIL	GPNHLEFIR	VWADYDPAAT	GRHYSEMYE	MLRIMAPPV	FGKCKPYRIA	972																		
rbB-I	E.....	DFA-F--V-	I--C-----	-----E	-----	-----YV	-----C	-S-ND-F-	-KH-S-L-	L--A-V-	1771																		
rbA-I	E.....	EFA-F--V-	I--C-----	-----E	-----	-----V	-----C	-S-ND-F-	-KH-S-L-	L--A-V-	1825																		
rbE-II	GQNHSEIR-T	DLA-V--V-	I--C-----	-----E	-----	-----V	-----C	-S-ND-F-	-KH-S-L-	L--A-V-	1738																		
rbC-I	ST-GETP-	SFAVF--I-	YM-CA--II-	-----W--	-----	-----K-	I--E--E-K	-K-KLDVVT	-L-RIQ-L-	-L-H-V-	1539																		
Unc-2	YKHLIRGMP	VARGD.TVHF	TTTLFALIRE	SLSIMGRPVE	EMDRA.DREL	RLTLKKIMPL	KAKKGMVDLV	VPPMHGLMFF	APP		1053																		
rbB-I	-R-V--	ISHEM--	-S-M--T	A-E--LA-AG	TKQRC-A--	-KRISV-AM	LQ-Q--TL--	---HK			1895																		
rbA-I	C-R-L-DL-	-D-N--	NS-M--T	A-D--IAKGG	ADKQCM-A--	-KEMQA--N	LSQ-Q--TL--	T-HK			1846																		
rbE-II	-R-VL-	---M--	-S-M--T	A-D--IAKGG	ADKQCM-S-	-QKTLA--H	LSQ-Q--LQ-	-MPK			1811																		
rbC-I	C-R-VS-	LNS--	-M-NA--	-S-M--T	A-R--	-TEG NLEQ-N--	-AII--KR	TSM--LQ--	-AG		1801																		



Mosaic type	Duplication absent in	Inferred loss
Egl-C/Kinker	PHBL or PHAL: 4 PHBR or PHAR: 1 all stain: 2	AB.pla or AB.plpp AB.prap or AB.prrp undetermined
Sluggish	P4: 2 all stain: 2	P1 undetermined
Wild-type	ASKL, ADLL, ASJL: 1 ASHL, PHAL: 2 PHAL or PHBL: 2 ASHL: 1 ASHR: 1	AB.a AB.plp AB.pla or AB.plpp AB.plpa AB.prap

FIG. 4 Site of *Unc-2* action in the control of egg laying. **a**, Pattern of *unc-2* mRNA expression in *C. elegans* hermaphrodites. Animals were fixed on polylysine slides, hybridized with a single-stranded digoxigenin (DIG)-labelled DNA probe, incubated with alkaline phosphatase-conjugated anti-DIG IgG (Boehringer), and stained with NBT/BCIP as described^{20,21}. Anti-sense probes were generated as described from two regions of the *unc-2* gene (probe A was complementary to the region encoding amino acids 46–215, probe B to the region encoding amino acids 459–642); a control sense probe was also generated (encoding amino acids 1–169). Using probe B, *unc-2* message was detected in both the HSN and VC neurons. Expression was also observed in the body wall muscle (mus) and in a subset of the neurons of the ventral nerve cord and the head (not shown). Similar staining was observed with probe A (not shown); no staining was observed with the sense probe. In principle we cannot rule out the possibility that these probes could detect the messages of other, closely related, calcium-channel proteins; however, Southern blots using probe B sequences do not detect significant hybridization to genes other than *unc-2*. **b** Analysis of putative *unc-2* mosaics. We identified potential mosaics using an *unc-2* mutant strain carrying an intact copy of the *unc-2* gene on an unstable free duplication (genotype: *him-5* (e1490); *unc-2*(e55) *osm-5*(p813); *yDp16*). Mitotic loss of the duplication during development results in mosaic animals containing clones of genetically mutant cells in an otherwise wild-type organism. Potential mosaics were identified on the basis of partial *unc-2* mutant phenotypes; for example, one class of animal showed kinking movement and constitutive egg laying (Egl-C), but was not sluggish, whereas a second class was sluggish, but showed coordinated movement and normal egg laying behaviour. Because the *C. elegans* cell lineage (outlined above) is invariant, and known in detail²², a cell-autonomous genetic marker can be used to infer the point of duplication loss, and thus to identify the cells that are genetically mutant. In this way we determined that most if not all of the Egl-C Kinkers had lost the duplication containing the functional *unc-2* gene in descendants of the cells AB.pl or AB.pr. Both these cells are precursors of neuronal cells, including the HSN, VC and adult motor neurons. In contrast, the Sluggish mosaics appeared to have lost the duplication in P1 or its descendants, which give rise to body wall and vulval muscle cells. The lower part indicates the cells in whose descendants the duplication was lost for each class of mosaics.

METHODS. We used the *osm-5* gene (like *unc-2*, *osm-5* is present on the duplication *yDp16* (ref. 23)) to score for the presence of the duplication in the cells of the amphid (ASHL, ASHR, ASJL, ASJR, ASKL, ASKR, ADLL and ADLR) and phasmid (PHAL, PHBL, PHAR, PHBR) sensilla; we also assayed for the presence of the duplication in the cell P4, the germline precursor cell, by scoring for transmission of the duplication to the progeny of the mosaic animal. The *osm-5* phenotype was scored as described¹⁶; briefly, animals were stained with Dil on 1.5% agar plates, and staining of the amphid and phasmid cells was visualized by fluorescence. The patterns of staining seen in the duplication-bearing strain indicated that a wild-type copy of *osm-5* was required autonomously in the sensory cells for staining with Dil (ref. 16); thus the *osm-5* genotype of a particular sensory cell could be determined.

Egl-C Kinkers were identified as kinkers that laid early (16-cell or earlier) embryos; wild-type mosaics laid normal or late-stage embryos and had approximately wild-type movement. **c**, Acute response of wild-type (N2), *egl-1*(n987) and *egl-1*(n987); *unc-2*(e55) strains to serotonin. Egg-laying assays were performed in liquid culture as described¹⁴; eggs were counted after 30 min incubation. Because the strains accumulate different numbers of eggs in the absence of drug, the dose/response curves were normalized by dividing the total eggs laid at a particular dose by the number laid by that population at the optimal dose. The following represents the mean rate of egg laying at the maximal dose: N2, 4.4 eggs per animal per hour (at 5 mg ml⁻¹); *egl-1*(n987), 10.9 (at 5 mg ml⁻¹); *egl-1*(n987); *unc-2*(e55), 9.0 (at 1.25 mg ml⁻¹). Each point represents a single trial of 10 animals; the line traces the mean of these three trials.

d, Model for the role of *Unc-2* in the control of egg laying: *unc-2* is expressed, and may act, in the VC and HSN neurons. An *unc-2* mutation increases serotonin responsiveness, and blocks desensitization, even in the absence of HSN. Because both the VCs and HSNs express a FMRFamide-like peptide that can potentiate serotonin response, one hypothesis to explain the *unc-2* mutant phenotype is that *Unc-2* is a subunit of a voltage-gated calcium channel that negatively regulates FMRFamide release. Serotonin adaptation may therefore involve activation of an *Unc-2*-dependent calcium influx in the VCs and/or HSNs, which inhibits the release of FMRFamide and thus decreases the responsiveness of the vulval muscles to serotonin.

the *egl-1* single mutant or the wild type (Fig. 4c). Moreover, whereas the *egl-1* single mutant still adapted to serotonin, the *egl-1*; *unc-2* double mutant was strongly adaptation defective (Fig. 2d). Thus *unc-2* mutants appear to lay eggs constitutively at least in part because their egg-laying muscles are hypersensitive and fail to adapt to endogenous serotonin. Where might the Unc-2 protein act to regulate egg laying? Mosaic analysis suggested that Unc-2 functions in neurons, yet the *egl-1* experiment demonstrated that Unc-2 does not require the HSNs to promote serotonin adaptation. An attractive hypothesis to explain these data is that Unc-2 modulates the release of FMRFamide, which can potentiate serotonin response from the VCs, and perhaps from the HSNs as well. Serotonin adaptation could occur if activation of the Unc-2 calcium channel causes an inhibition of FMRFamide release and thus a decrease in serotonin response (Fig. 4d).

In summary, we have shown that a voltage-gated calcium channel appears to be required for adaptation to dopamine and serotonin, and for determining the postsynaptic threshold for serotonin response. The *unc-2* gene, which encodes the α -1 subunit of this channel, is expressed in neurons that control egg-laying behaviour. We propose that, in these neurons, *unc-2* may participate in serotonin adaptation by modulating the serotonin response, perhaps by controlling the release of a neuropeptide. The *unc-2* message is also expressed in several other neuronal and body muscle cells that may control response to dopamine; determination of the behavioural functions of *unc-2* in these

cells may help to elucidate the mechanisms underlying dopamine adaptation. □

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Involvement of an ICE-like protease in Fas-mediated apoptosis

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FAS is a type-I membrane protein that transduces an apoptotic signal^{1,2}. Binding of Fas ligand or agonistic anti-Fas antibody to Fas kills the cells by apoptosis³. Studies in the nematode *Caenorhabditis elegans* have suggested that proteases such as interleukin-1 β -converting enzyme (ICE) or the product of the *C. elegans* cell-death gene *ced-3* are involved in apoptotic signal transduction⁴. The activity of ICE can be inhibited by the product of *crmA*, a cytokine-response modifier gene encoded by cowpox virus^{5–7}. We report here that expression of *crmA* inhibits cytotoxicity induced by anti-Fas antibody or tumour necrosis factor (TNF). We have found a specific ICE inhibitor tetrapeptide (acetyl-Tyr-Val-Ala-Asp-chloromethylketone)^{8,9} that also prevents apoptosis induced by anti-Fas antibody. These results suggest an involvement of an ICE-like protease in Fas-mediated apoptosis and TNF-induced cytotoxicity.

Expression plasmids encoding *crmA*¹⁰ and human *fas*¹ were introduced into Rat-2 (rat fibroblast) cells together with the neomycin-resistance gene. Among G418-resistant transformants, the Fas-expressing transformants were selected by flow cytometry analysis using mouse anti-human Fas antibody. Three clones (33, 45 and 48) were found to express human Fas on the cell surface (data not shown). Immunoprecipitation using anti-human Fas antibody confirmed the expression of Fas in

these transformants (Fig. 1a). As found previously¹, immunoprecipitation gave two bands (M_r 43K and 50K) for human Fas, which may reflect different degrees of glycosylation. The expression of *crmA* was then analysed by RNase protection assay using a ³²P-labelled RNA fragment carrying the *crmA* gene (nucleotides 1,174 to 1,468). The control *crmA* RNA produced by *in vitro* transcription gave protected bands of 280, 250 and 150 bases (Fig. 1b). The upper band was of the size expected, but the other two bands may have been products of digestion at AU-rich regions of the *crmA* RNA. The same protected bands were observed with messenger RNAs from clones 33 and 48, but no such bands were detected with mRNA from the parental Rat-2 cells and clone 45.

The parental Rat-2 cell line and its transformants were then treated with the agonistic anti-human Fas antibody. As shown in Fig. 2a, the anti-Fas antibody had no effect on Rat-2 cells. However, clone 45, which expressed human Fas but not CrmA, was killed within 14 hours by the anti-Fas antibody in a dose-dependent manner in the presence of 50 ng ml⁻¹ actinomycin D. Almost all cells died within 10 hours of treatment with 1 μ g ml⁻¹ of the anti-Fas antibody (Fig. 2b). However, the transformant clones 33 and 48 that expressed CrmA were resistant to the cytotoxic activity of the anti-human Fas antibody, and even survived incubation for 10 hours with 1 μ g ml⁻¹ of anti-Fas antibody. The inhibition of Fas-mediated apoptosis by CrmA was dose dependent, that is, the transformed clones expressing small amounts of *crmA* mRNA were weakly protected against Fas-mediated apoptosis (data not shown).

TNF has cytotoxic activity in various cell lines. To examine whether an ICE-like protease is also involved in TNF-induced cytotoxicity, Rat-2 cells and its transformants were treated with TNF. The Rat-2 cells were killed by 250 ng ml⁻¹ of mouse TNF in the presence of 12.5 ng ml⁻¹ actinomycin D (Fig. 2c), although this process was much slower than Fas-mediated cytotoxicity (all cells were killed in ~60 hours). Clone 45, which expresses Fas but not CrmA, was killed by TNF treatment as efficiently as were the parental Rat-2 cells, but clones 33 and 48,

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FIG. 1 *a*, Immunoprecipitation of human Fas. The parental Rat-2 (lane 1), and its transformant clones 33 (lane 2), 45 (lane 3) and 48 (lane 4) were biotinylated, lysed in an NP-40-containing buffer, preabsorbed with normal mouse IgG and immunoprecipitated with mouse anti-human Fas antibody as described¹³. The immunoprecipitates were electrophoresed through a polyacrylamide gel, blotted onto a nitrocellulose filter and detected using the ECL system (Amersham). The sizes of marker proteins are shown on the left. *b*, RNase protection assay. ³²P-labelled antisense *crmA* RNA (2×10^5 c.p.m.) was hybridized with 1.0 μ g poly(A)⁺ RNA from parental Rat-2 (lane 1) and its transformant clones 33 (lane 2), 45 (lane 3), 48 (lane 4), or with control sense *crmA* RNA (lane 5), digested with a mixture of RNase A and RNase T1, and electrophoresed on a 6% polyacrylamide gel containing 8.3 M urea as described²⁸. Undigested probe RNA (lane 6) and size-marker DNAs were electrophoresed in parallel. Marker DNA sizes are shown on the left, and the protected bands are indicated by arrows on the right.

METHODS. The 1.5-kilobase (kb) cowpox virus DNA fragment encoding CrmA¹⁰, provided by D. J. Pickup, was inserted into mammalian expression vector pEF-BOS²⁹ to generate pEFcrmA. Rat-2 cells (JCRB IFO 50282) were maintained in Dulbecco's modified Eagle's medium containing 5% fetal calf serum. The cells were transfected with 10 μ g pEFcrmA, 10 μ g pEFF58 (ref. 1) and 0.5 μ g pSTneoB by electroporation as described¹, and G418-resistant transformants were selected by culturing cells in medium containing 900 μ g ml⁻¹ G418 for 3 weeks. For the RNase protection assay, the 1.5-kb *Crma* fragment was inserted at the *Eco*RI site of pBluescript

(pBScrmA), digested with *Pvu*II, and the ³²P-labelled antisense RNA corresponding to the C-terminal part of CrmA was synthesized in an *in vitro* transcription system using T7 RNA polymerase (Promega) and [α -³²P]CTP (Amersham). To prepare the control sense *Crma* RNA, pBScrmA was digested with *Hind*III, and transcribed *in vitro* using T3 RNA polymerase (Promega). Poly(A)⁺ RNA was prepared from Rat-2 and its transformants using a mRNA purification kit (Pharmacia).

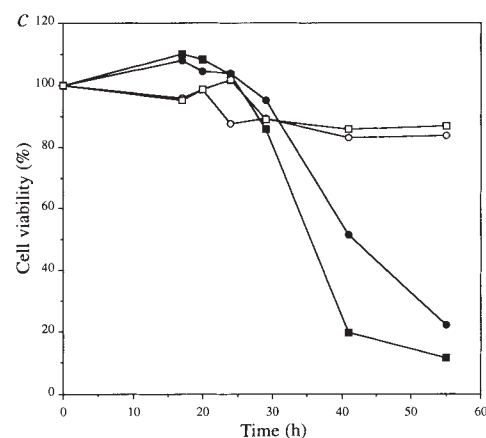
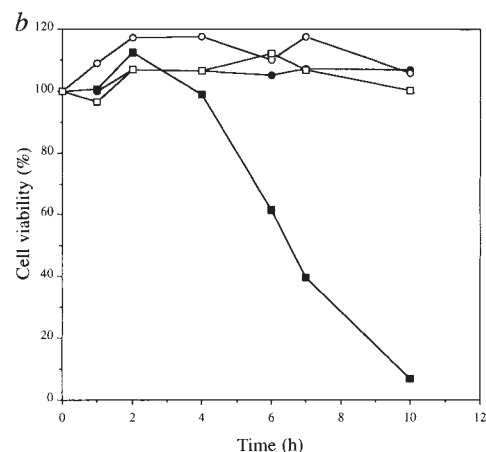
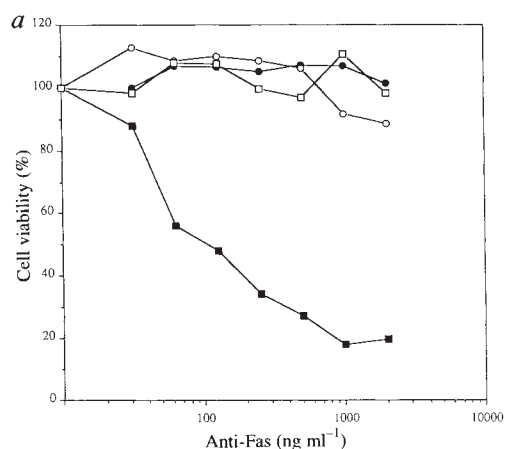


FIG. 2 Effect of CrmA on anti-Fas antibody-induced (*a*, *b*) and TNF-induced (*c*) apoptosis. *a*, The cells were treated for 14 hours with various concentrations of anti-human Fas antibody (CH-11; Medical and Biological Laboratories) in the presence of 50 ng ml⁻¹ actinomycin D. *b*, The cells were treated for various periods with 1.0 μ g ml⁻¹ of anti-Fas antibody in the presence of 50 ng ml⁻¹ actinomycin D. *c*, After preincubation with 12.5 ng ml⁻¹ actinomycin D for 4 hours, the cells were treated for various periods with 250 ng ml⁻¹ murine TNF in the presence of 12.5 ng ml⁻¹ actinomycin D. The viable cells were quantified by staining with crystal violet as described¹. Filled circles, the parental Rat-2 cell; open circles, transformant clone 33; filled squares, clone 45; open squares, clone 48.

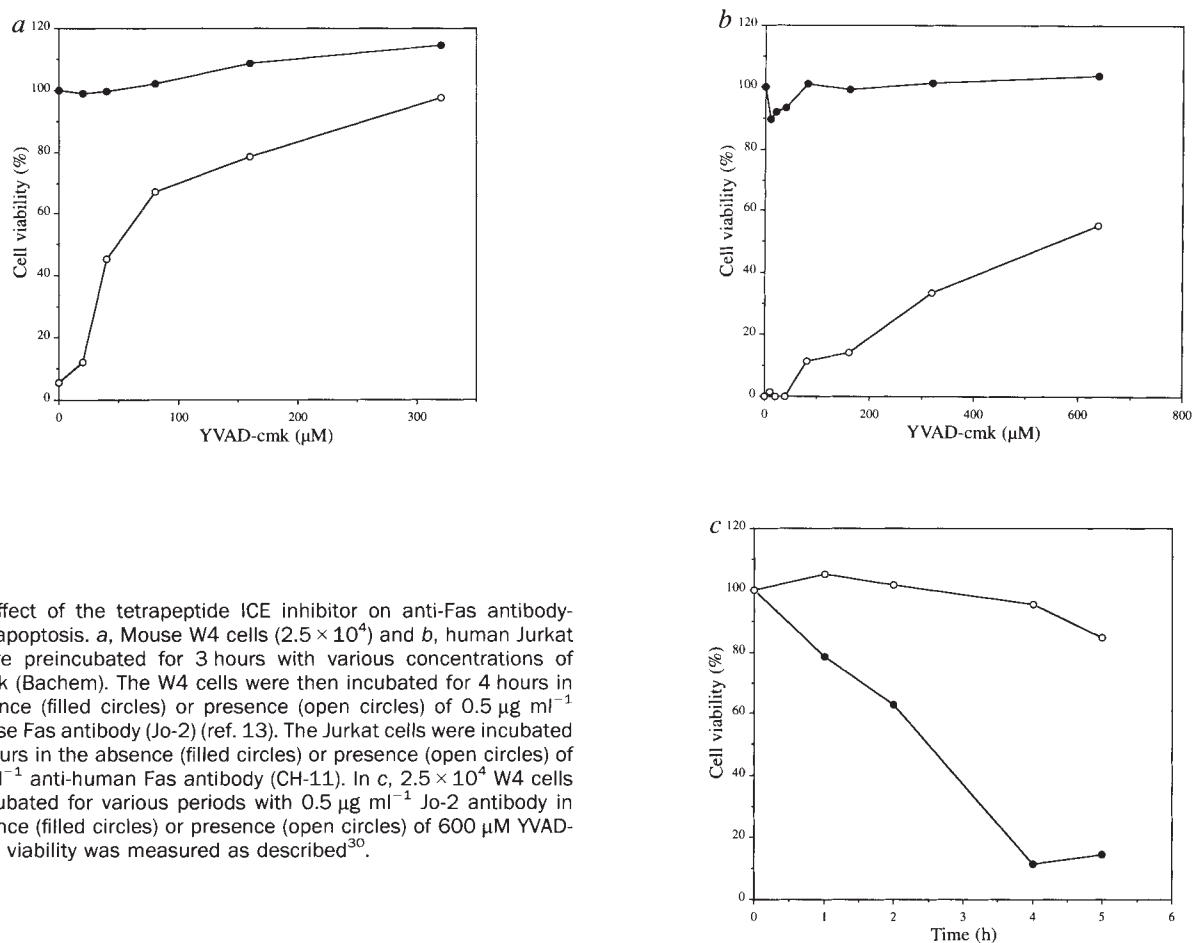


FIG. 3 Effect of the tetrapeptide ICE inhibitor on anti-Fas antibody-induced apoptosis. *a*, Mouse W4 cells (2.5×10^4) and *b*, human Jurkat cells were preincubated for 3 hours with various concentrations of YVAD-cmk (Bachem). The W4 cells were then incubated for 4 hours in the absence (filled circles) or presence (open circles) of $0.5 \mu\text{g ml}^{-1}$ anti-mouse Fas antibody (Jo-2) (ref. 13). The Jurkat cells were incubated for 23 hours in the absence (filled circles) or presence (open circles) of $1.0 \mu\text{g ml}^{-1}$ anti-human Fas antibody (CH-11). In *c*, 2.5×10^4 W4 cells were incubated for various periods with $0.5 \mu\text{g ml}^{-1}$ Jo-2 antibody in the absence (filled circles) or presence (open circles) of $600 \mu\text{M}$ YVAD-cmk. Cell viability was measured as described³⁰.

which expressed CrmA, were resistant to TNF-induced cytotoxicity. These results indicate that an ICE-like protease is involved not only in Fas-mediated apoptosis but also in TNF-induced cytotoxicity. It has previously been shown¹¹ that TNF-induced cytotoxicity is partly inhibited by a serine-protease inhibitor, plasminogen-activator inhibitor type 2. Because plasminogen-activator inhibitor type 2 is a member of the serpin superfamily to which CrmA belongs⁷, it is possible that the plasminogen activator inhibitor also inhibits the ICE-like protease by 'cross-class' interaction¹².

The cytotoxicity of the anti-Fas antibody and TNF in Rat-2 cells was seen only in the presence of actinomycin D. We tried to express *crmA* in other cell lines, such as mouse W4 (ref. 13) or human Jurkat cells¹⁴, which can be killed by anti-Fas antibody without actinomycin D. However, stable transformants constitutively expressing CrmA were not obtained because of the strong growth-inhibitory effect of CrmA in these cell lines. To overcome this problem, and to confirm the involvement of an ICE-like protease in Fas-mediated apoptosis in other cells, we used a tetrapeptide of acetyl-Tyr-Val-Ala-Asp-chloromethylketone (YVAD-cmk), which works as a specific inhibitor for ICE or ICE-like proteases^{8,9}. Treatment of W4 cells with $0.5 \mu\text{g ml}^{-1}$ anti-mouse Fas antibody or Jurkat cells with $1.0 \mu\text{g ml}^{-1}$ anti-human Fas antibody killed the cells (Fig. 3). Addition of YVAD-cmk inhibited the killing process in a dose-dependent manner. The kinetic analysis of the death process of W4 in the presence of $600 \mu\text{M}$ YVAD-cmk confirmed its inhibitory activity against the anti-Fas antibody-induced apoptosis (Fig. 3c). However, as found with purified ICE⁹, other protease inhibitors, such as TPCK, PMSF and E-64, had no effect on Fas-mediated apoptosis (data not shown).

TNF type-I receptor (TNFRI) and Fas carry a similar killing domain in the cytoplasmic region^{15,16} and we suggest here that

both receptors use an ICE-like molecule to kill the cells. This indicates that the apoptotic mechanism is well conserved during evolution from nematodes to mammals. Programmed cell death in nematodes is inhibited by CED-9 (ref. 17). Significant inhibition of Fas-mediated apoptosis by Bcl-2 (ref. 18), a mammalian homologue of *ced-9*, is in agreement with this. It is possible that signalling molecules immediately downstream of the receptors are distinct between Fas and TNFRI^{19,20} but these pathways would eventually lead to the activation of an ICE-like protease. Because at least three ICE or ICE-like proteases exist in mammalian cells^{9,21-23}, it would be necessary to determine which ICE-like protease is involved in Fas- and TNFRI-mediated apoptosis. ICE is a tetrameric protein, consisting of $(p20)_2/(p10)_2$ in the cytoplasm²⁴. Because Fas-mediated apoptosis occurs in the presence of inhibitors of RNA or protein synthesis, the activation of an ICE-like protease during Fas-mediated apoptosis is probably a post-transcriptional event. A signal(s) from Fas or TNFRI may activate the processing of the precursor of an ICE-like protease⁹, or inactivate the CrmA-like cellular protein. The Fas ligand works as an effector of cytotoxic T-lymphocytes (CTL)^{25,26} and it is possible that the Fas system is involved in CTL-mediated disease, such as fulminant hepatitis or AIDS²⁷. If so then inhibitors of ICE such as the YVAD-cmk, may be of clinical benefit.

Note added in proof: Tewari and Dixit have recently reported that the *crmA* product can inhibit Fas- and TNF-induced apoptosis (*J. biol. Chem.* **270**, 3255-3260 (1995)). □

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Requirement of an ICE/CED-3 protease for Fas/APO-1-mediated apoptosis

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THE Fas/APO-1 receptor is one of the major regulators of apoptosis^{1–7}. We report here that Fas/APO-1-mediated apoptosis requires the activation of a new class of cysteine proteases, including interleukin-1 β -converting enzyme (ICE)^{8–10}, which are homologous to the product of the *Caenorhabditis elegans* cell-death gene *ced-3* (refs 11, 12). Triggering of Fas/APO-1 rapidly stimulated the proteolytic activity of ICE. Overexpression of ICE, achieved by electroporation and microinjection, strongly potentiated Fas/APO-1-mediated cell death. In addition, inhibition of ICE activity by protease inhibitors, as well as by transient expression of the pox virus-derived serpin inhibitor CrmA or an antisense ICE construct, substantially suppressed Fas/APO-1-triggered cell death. We conclude that activation of ICE or an ICE-related protease is a critical event in Fas/APO-1-mediated cell death.

The signal transduction pathway elicited by Fas/APO-1 is almost completely unknown. Initiation of apoptosis may involve a new class of cysteine proteases, including the product of the *C. elegans* cell-death gene *ced-3*, mammalian interleukin-1 β -converting enzyme (ICE) and the related proteases Nedd-2/Ich-1, prICE and CPP-32 (refs 11–17). Overexpression of CED-3, ICE or Nedd-2/Ich-1 in Rat-1 fibroblasts has been shown to result in apoptotic cell death^{12,15}. We therefore investigated whether Fas/APO-1-mediated apoptosis involved an ICE-related proteolytic activity. In L929-APO-1 cells¹⁸ or B-lymphoblastoid SKW 6.4 cells, apoptosis triggered by the agonistic monoclonal antibody anti-APO-1 was strongly inhibited by the ICE inhibitor YVAD-CHO, a tetrapeptide aldehyde ($K_i = 0.76$ nM)⁸ (Fig. 1a). Inhibition was also observed with the protease inhibitor dichloroisocoumarin, but other serine protease inhibitors, such as

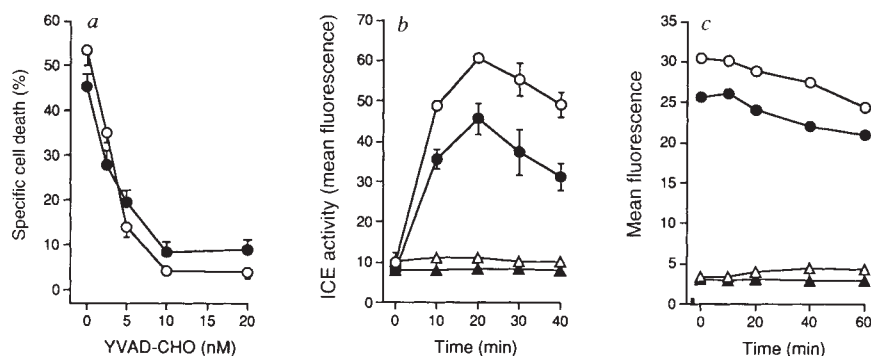
PMSF and leupeptin, calpain inhibitors and the cysteine protease inhibitor E-64 were not effective (data not shown). In addition, ICE-like proteolytic activity was readily induced by Fas/APO-1 ligation (Fig. 1b). The fluorogenic ICE substrate DABCYL-YVADAP-EDANS, which contains the cleavage site of the interleukin-1 β precursor¹⁹, was cleaved after treatment of permeabilized cells with anti-APO-1, but no effects were detected using classical cysteine or serine protease-specific substrates (Fig. 1b, c).

To explore further the participation of ICE in Fas/APO-1-mediated apoptosis, ICE was overexpressed using several techniques. First, murine ICE complementary DNA was microinjected into nuclei of L929-APO-1 cells. After treatment with anti-APO-1, apoptotic cells could be recognized as round-shaped cells revealing membrane blebbing and cytoplasmic condensation. When cells microinjected with ICE cDNA were treated with a suboptimal dose of anti-APO-1, a nearly threefold increase in the number of apoptotic cells was detected compared with cells microinjected with the empty vector alone (Fig. 2a). In contrast, microinjection of vaccinia virus-derived *crmA* cDNA, the product of which inhibits ICE activity by forming a serpin-like pseudosubstrate^{20–22}, significantly suppressed anti-APO-1-induced cell death. These observations suggested the involvement of ICE or an ICE-related protease in the Fas/APO-1 signalling pathway. Although ICE is the only protease known to be inhibited by CrmA, it is possible that a related protease with similar substrate specificity was inhibited by CrmA. To investigate more specifically the role of ICE, we further included an antisense ICE construct. ICE, *crmA* and antisense ICE cDNAs were overexpressed by electroporation, which resulted in transfection efficiencies of more than 80% as assessed by reporter gene plasmids. Figure 2b shows that apoptosis was increased by transient expression of ICE after anti-APO-1 treatment, whereas apoptosis induced in their *crmA*- or antisense-ICE-transfected counterparts was reduced. The effects on apoptosis were further evaluated in L929-APO-1 cells after cotransfection with the *lacZ* gene as a marker of gene expression (Fig. 3). In comparison with cells transfected with the vector control, the percentage of round apoptotic cells out of the total number of blue-stained cells was substantially increased in ICE cDNA-transfected cells after anti-APO-1 treatment. As in the previous experiments, no significant difference in cell viability of L929-APO-1 cells was observed without Fas/APO-1 activation. This is in apparent contrast to other cell types undergoing apoptosis by overexpression of ICE alone^{12,15}. In line with the previous data, transient overexpression of CrmA or antisense-ICE resulted in an inhibition of anti-APO-1-induced apoptosis of ~50% (Fig. 3g).

These data indicate that ICE plays a role in the induction of apoptosis mediated by Fas/APO-1. Although it cannot be excluded that other ICE-related proteases may also be involved, the antisense experiments suggest that ICE is important. Because ICE is structurally and functionally related to the nematode

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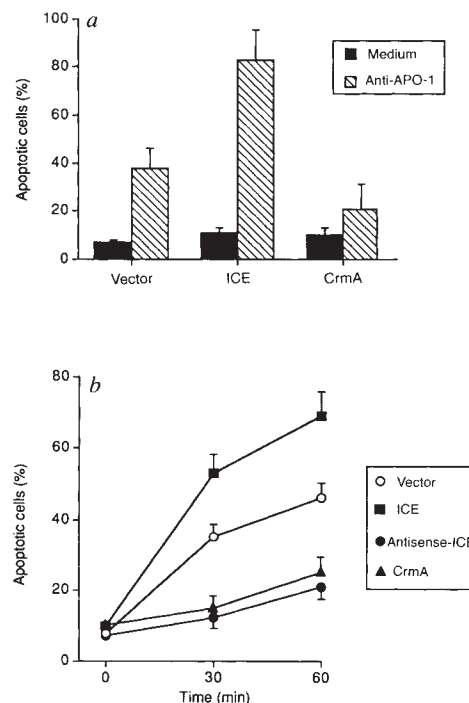
FIG. 1 Involvement of an ICE-related protease activity in Fas/APO-1-induced apoptosis. **a**, Dose-dependent inhibition of Fas/APO-1-triggered apoptosis by the tetrapeptide ICE inhibitor YVAD-CHO. L929-APO-1 (open circles) and SKW 6.4 cells (filled circles) were cultured as described^{23,24}, made permeable by a short hypotonic shock and then incubated with the indicated concentrations of the inhibitor for 30 min. Cells were treated in medium containing actinomycin D with $1 \mu\text{g ml}^{-1}$ anti-APO-1 for an additional 4 hours (L929-APO-1 cells, 400 ng ml^{-1} anti-APO-1; SKW 6.4 cells, 50 ng ml^{-1} anti-APO-1). Apoptosis was assessed by propidium iodide uptake ($2.5 \mu\text{g ml}^{-1}$) and fluorescence-activated cell sorting (FACS) analysis. Data of specific cell death (cell death in the presence of anti-APO-1 minus cell death in the absence of anti-APO-1) were obtained from triplicate experiments. Spontaneous cell death in the absence of anti-APO-1 was less than 10%. Similar results were obtained by measuring DNA fragmentation with the dye Hoechst 33342 (Molecular Probes, Eugene, OR)²⁵. **b**, Stimulation of ICE-like proteolytic activity by anti-APO-1. L929-APO-1 and SKW 6.4 cells were cultured at 7×10^4 in 35-mm plates in medium containing actinomycin D and either left untreated or treated with anti-APO-1 ($1 \mu\text{g ml}^{-1}$) for the indicated time points. Cells were made permeable 10 min before collection by using 0.05% digitonin and incubated with $20 \mu\text{M}$ of the fluorogenic ICE substrate DABCYL-YVADAP-EDANS¹⁹ (Bachem, Bubendorf, Switzerland). Cells were collected with a rubber policeman and analysed by FACS analysis using an excitation wavelength of 360 nm and emission wavelength of



488 nm. Open circles and triangles represent L929-APO-1 cells plus/minus anti-APO-1, respectively; filled circles and triangles represent SKW 6.4 cells plus/minus anti-APO-1, respectively. **c**, Effect of anti-APO-1 on other protease activities: L929-APO-1 and SKW 6.4 cells were treated as described in **a** and incubated with the serine (trypsin-like) protease substrate Bz-Val-Gly-Arg-AMC (open triangles, L929-APO-1 cells; filled triangles, SKW 6.4 cells) or the cysteine protease-specific substrate (CBZ-Phe-Arg)₂-R110 (open circles, L929-APO-1 cells; filled circles, SKW 6.4 cells). Cleavage of the substrates was measured by FACS analysis using excitation and emission wavelengths of 366 nm and 460 nm, respectively, for Bz-Val-Gly-Arg-AMC ($30 \mu\text{M}$; Bachem) and 488 nm and 530 nm for (CBZ-Phe-Arg)₂-R110 ($30 \mu\text{M}$; Molecular Probes).

FIG. 2 Effect of transient expression of ICE, *crmA* and antisense ICE cDNA on Fas/APO-1-mediated apoptosis by **a**, microinjection and **b**, electroporation.

METHODS. Microinjection: 8×10^5 L929-APO-1 cells were seeded in 60-mm tissue dishes. After overnight incubation, cell nuclei were microinjected with expression plasmids encoding murine ICE or vaccinia virus-derived CrmA. Cell injections were performed with an automatic microinjection system (Zeiss ALS) equipped with glass micropipettes which had been loaded with $1 \mu\text{l}$ of the appropriate DNA diluted to about $0.25 \mu\text{g ml}^{-1}$ with 2.5% FITC-labelled dextran. After 20 hours of further incubation, cells were either left untreated or incubated with $0.5 \mu\text{g ml}^{-1}$ anti-APO-1 medium containing actinomycin D. After 45 min, cells were fixed with 2.5% glutaraldehyde and inspected microscopically. Cells were regarded as apoptotic when they revealed membrane blebbing and/or a condensed cell nucleus. At least 180 cells were analysed for each condition in three independent experiments. A murine ICE cDNA was isolated by polymerase chain reaction after reverse transcription of RNA using EL4/c mRNA and oligo(dT) primers for the first-strand synthesis. A 1,322-base-pair (bp) PCR product was isolated using the primers ATCGGATCCAGCATGGCTGACAAGATCCTGAGG (plus strand) and CGGCCTCGAGCATCATCTAAGGAAGTATTGGC (minus strand) and used as a probe to screen a EL4/13 cDNA expression library cloned into pCAGGS. The pCAGGS vector was provided by J. Miyazaki and contained a CMV/ β -actin promoter²⁶. A 1,387-bp full-length ICE cDNA clone was isolated and its sequence confirmed by double-stranded DNA sequencing. The biological activity of the ICE cDNA product was checked after cotransfection of a pro-interleukin-1 β expression plasmid (gift from J. F. DeLamarter) in COS cells. The *crmA* cDNA was obtained from vaccinia virus DNA after standard PCR with the primers 5'-GCGAAGCTTACACGACCAATATCGATTACTA-3' and 5'-CGCCATGGTTAACAATTAGTTGTCGGAGAG-3'. The PCR product was cloned as a HindIII/KpnI fragment in pSV25S, an expression plasmid containing the simian virus 40 (SV40) early promoter. All plasmids were purified by caesium chloride density gradient centrifugation. For electroporation, L929-APO-1 cells were washed in Tris-buffered saline (TBS), resuspended at 1×10^6 cells per 0.4 ml TBS and equilibrated and transfected with $20 \mu\text{g}$ of expression plasmids using a Bio Rad electroporator (960 μF , 230 V). After electroporation, cells were seeded at 1×10^6 cells per well in 6-well plates. Dead cells were removed after 16 h by a washing step in culture medium. Cells were treated for the indicated times with anti-APO-1 ($1 \mu\text{g ml}^{-1}$) in medium containing actinomycin D. Apoptosis was measured by



FACS analysis using Hoechst 33342 (ref. 25). Data are given as mean percentage cell death from four experiments with duplicate samples. An antisense-ICE construct was obtained after cloning a 320-bp EcoRI fragment of ICE cDNA containing 48 bp of the 5'UTR and the first 255 bp of the ICE open reading frame in reverse direction into the pCAGGS vector.

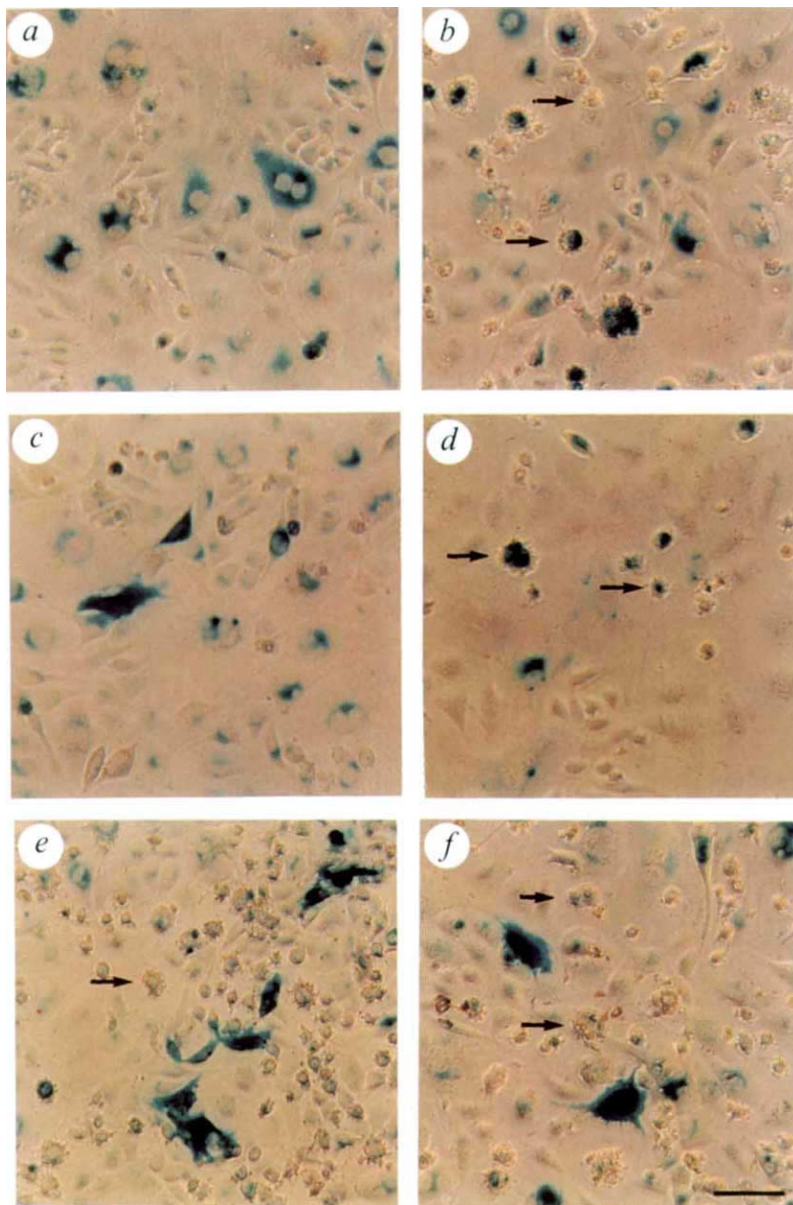
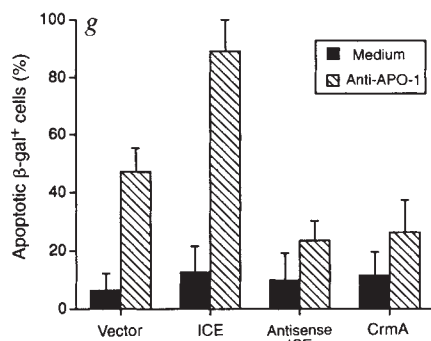


FIG. 3 X-Gal staining of L929-APO-1 cells expressing ICE, antisense ICE, *crmA* cDNA or empty vector after cotransfection with *lacZ*. L929-APO-1 cells were cotransfected with an expression vector encoding *lacZ*, together with a vector control (a, b), ICE (c, d), antisense ICE (e) or *crmA* cDNA (f). Cells were either left untreated in medium containing actinomycin D (a, c) or treated with anti-APO-1 (b, d-f) 24 h after transfection and then stained for 12 h with X-Gal solution. The scale bar represents 20 μ m. Examples of apoptotic cells are indicated by arrows. g, Data of transfections obtained from two experiments. The values give the percentage of blue apoptotic cells out of the total number of blue cells. At least 450 transfected cells were counted for each condition. Faintly stained cells were not included.

METHODS. The day before transfection, cells were seeded in 35-mm dishes at 7×10^4 cells and 0.5 ml culture medium. For each well, 400 ng of the *lacZ* eukaryotic expression construct CMV- β -gal (Clontech) and 1,200 ng of the ICE, antisense-ICE or *crmA* expression plasmid were used. Transfections were performed by liposome-mediated gene transfer using the DOTAP reagent for 24 h according to the instructions of the manufacturer (Boehringer Mannheim). After transfection, cells were washed in culture medium and treated with anti-APO-1 ($1 \mu\text{g ml}^{-1}$) for 45 min. To detect gene expression, cells were fixed with 1% glutaraldehyde for 5 min, rinsed once with PBS and stained for 10–12 h in X-Gal buffer containing 5 mM $\text{K}_4\text{Fe}(\text{CN})_6$, 5 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 2 mM MgCl_2 and 1 mg ml^{-1} 5-bromo-4-chloro-3-indoxyl- β -galactoside.



gene *ced-3*, our data imply that Fas/APO-1-mediated apoptosis follows a highly conserved signalling pathway. □

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Allosteric transition intermediates modelled by crosslinked haemoglobins

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THE structural end-points of haemoglobin's transition from its low-oxygen-affinity (T) to high-oxygen-affinity (R) state, have been well established by X-ray crystallography¹⁻⁷, but short-lived intermediates have proved less amenable to X-ray studies. Here we use chemical crosslinking to fix these intermediates for structural characterization. We describe the X-ray structures of three haemoglobins, $\alpha_2\beta^1S^{82}\beta$, $\alpha_2\beta^1Tm^{82}\beta$ and $\alpha_2\beta^{1,82}Tm^{82}\beta$, which were crosslinked between the amino groups of residues β Val1 and β Lys82 by 3,3'-stilbenedicarboxylic acid (S) or trimesic acid (Tm) while in the deoxy state, and saturated with carbon monoxide before crystallization. $\alpha_2\beta^1S^{82}\beta$, which has almost normal oxygen affinity, is completely in the R-state conformation; however, $\alpha_2\beta^1Tm^{82}\beta$ and $\alpha_2\beta^{1,82}Tm^{82}\beta$, both of which have low oxygen affinity, have been prevented from completing their transition into the R state and display many features of a transitional intermediate. These haemoglobins therefore represent a snapshot of the nascent R state.

The crosslinked haemoglobins were prepared by reacting human deoxyhaemoglobin with the diacyl bis(methylphosphate)

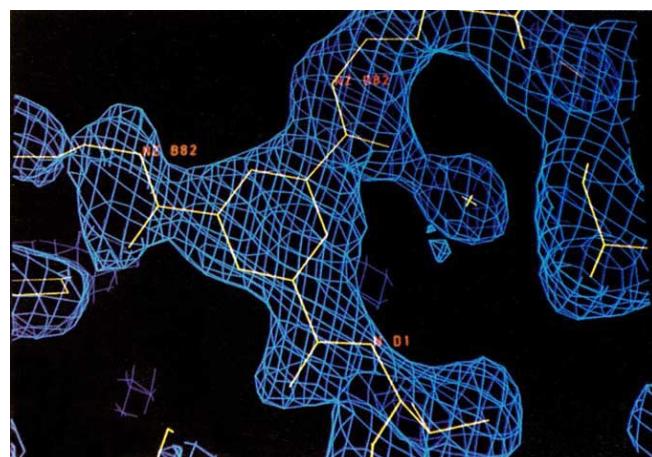


FIG. 1 $2F_o - F_c$ electron density map of the triply linked $\alpha_2\beta^{1,82}Tm^{82}\beta$ haemoglobin contoured at 1.0σ . The atoms are represented as yellow sticks and the electron density map is indicated in blue²⁵. The cross represents the location of a water molecule that is hydrogen-bonded to the carbonyl group of the crosslinker. Shown is the trimesoyl crosslinker bonded to the ϵ -NH₂ of β_1 Lys82 (N ζ D82), the α -NH₂ of β_1 Val1 (N D1) and the ϵ -NH₂ of β_2 Lys82 (N ζ B82), providing the first example of a rationally designed triply linked protein.

derivatives of 3,3'-stilbenedicarboxylic acid (S)⁸ or trimesic acid (Tm)⁹ and were crystallized in the carbonmonoxide (CO) form by the method of Perutz¹⁰. $\alpha_2\beta^1S^{82}\beta$, which has a slightly higher O₂ affinity than haemoglobin A (HbA) and high cooperativity (Table 1), crystallizes isomorphously with respect to CO-HbA^{1,7}. The structure has been refined¹¹ to a crystallographic *R*-factor of 14.6% at 2.4 Å resolution (Table 1). Both $\alpha_2\beta^1Tm^{82}\beta$ and $\alpha_2\beta^{1,82}Tm^{82}\beta$ show markedly reduced O₂ affinity compared with HbA, high cooperativity, and crystallize in space group *C2* (Table 1). The structure of $\alpha_2\beta^1Tm^{82}\beta$ was solved by molecular replacement¹² and found to contain a tetramer per asymmetric unit. Refinement converged to an *R*-factor of 15.0% at 1.8 Å

FIG. 2 Stereo view of the overlay of the $\alpha_1\beta_1$ interface of deoxyhaemoglobin (blue α_1 trace), $\alpha_2\beta^1Tm^{82}\beta$ (yellow α_1 trace), $\alpha_2\beta^{1,82}Tm^{82}\beta$ (green α_1 trace) and $\alpha_2\beta^1S^{82}\beta$ (white α_1 trace) onto CO-HbA (red α_1 trace) using the method of Baldwin and Chothia¹. This figure dramatically illustrates the quaternary changes that occur in the T-to-R transition. In this overlay method, the regions in which no structural changes occur in going from T to R are used as a reference frame and include residues α 30– α 36 (B helix); α 102– α 113 (G helix); α 117– α 127 (H helix); β 30– β 36 (B helix); β 51– β 55 (D helix); β 107– β 132 (G and H helices). After overlaying this region of the $\alpha_1\beta_1$ dimers, a rotation of 13.6° is required to bring the $\alpha_2\beta_2$ interfaces of the deoxy- and CO-HbA into coincidence. The TmHbs appear to be intermediates in this transition as they have rotated only 7.4° and 8.5° about an axis of rotation nearly coincident to that used by native CO-HbA. Their translation components, approximately half (0.44) of that of native Hb, are also intermediate. No additional rotation or translation is required for the $\alpha_2\beta^1S^{82}\beta$ haemoglobin, consistent with its complete R-state conformation. Coordinates used in all analyses were taken from refs 7 (CO-HbA) and 2 (deoxyhaemoglobin).

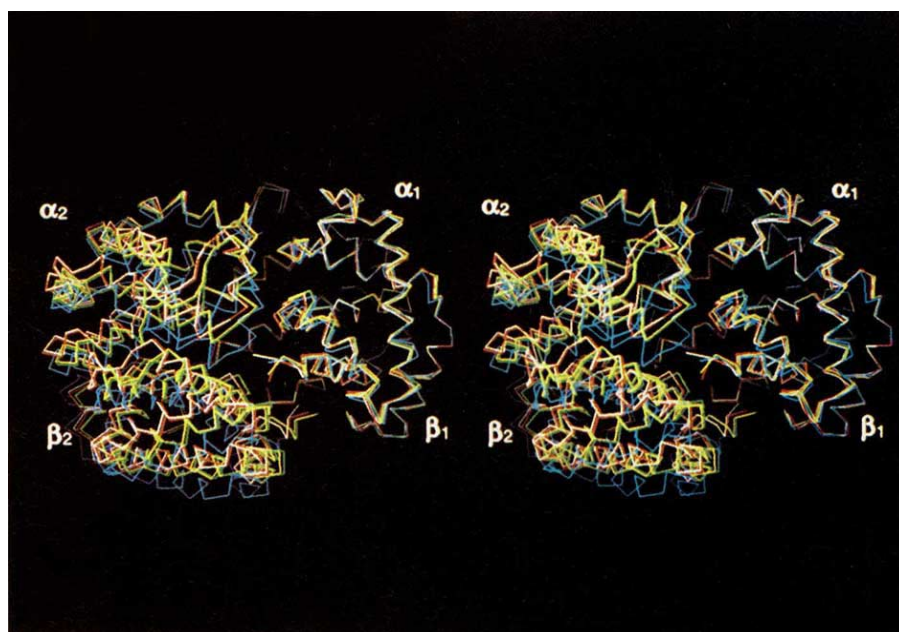
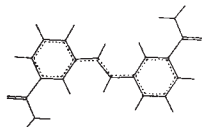
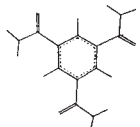
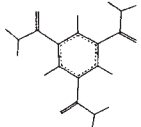


TABLE 1 Summary of selected biochemical and crystallographic data

Crosslinked haemoglobin	$\alpha_2\beta^1S^{82}\beta$	$\alpha_2\beta^1Tm^{82}\beta$	$\alpha_2\beta^{1,82}Tm^{82}\beta$
Crosslinker			
P_{50} (mmHg)	3.4 ⁸	17.1 ⁸	18.1 ⁹
Hill coefficient	2.6 ⁸	2.7 ⁸	2.6 ⁹
Space group	$P4_12_12$	C2	C2
Cell dimensions (Å)	$a=b=54.13$ $c=196.23$	$a=104.43$, $b=72.16$ $c=88.03$ $\beta=108.25^\circ$	$a=104.43$, $b=72.16$ $c=88.03$ $\beta=108.25^\circ$
$\alpha\beta$ dimer per ASU	1	2	2
Data collection			
Resolution (Å)	2.4	1.8	1.8
Number of reflections	9,807	48,608	42,152
R_{sym} (%)	3.6	6.3	3.0
Number of atoms	2,300	4,576	4,577
Number of solvent molecules	110	393	429
Refinement			
R -factor (%)	14.6	15.0	13.5
Deviations from ideality (r.m.s.)			
Bond distances (Å)	0.019	0.010	0.017
Bond angles (deg)	2.90	2.34	3.01

$\alpha_2\beta^1S^{82}\beta$, $\alpha_2\beta^1Tm^{82}\beta$ and $\alpha_2\beta^{1,82}Tm^{82}\beta$ were crosslinked in their deoxy forms as described^{8,9} by reacting human deoxyhaemoglobin with the diacyl bis(methylphosphate) derivatives of 3,3'-stilbenecarboxylic acid (S) or trimesic acid (Tm). $\alpha_2\beta^1S^{82}\beta$ and $\alpha_2\beta^1Tm^{82}\beta$ are crosslinked through the α -NH₂ of β_1 Val1 to the ϵ -NH₂ of β_2 Lys82, where beta subscripts 1 and 2 indicate different β -subunits only. $\alpha_2\beta^{1,82}Tm^{82}\beta$ is crosslinked through the α -NH₂ of β_1 Val1 and the ϵ -NH₂ of β_1 Lys82 to the ϵ -amino group of β_2 Lys82. The crosslinkers are depicted in their amide forms. The negative charge of the methyl phosphate leaving groups serves to target the crosslinkers to the cationic 2,3-bis-phosphoglycerate (BPG)-binding pocket, where β Lys82 and β Val1 are located. Comparison of the P_{50} values and Hill coefficients of the crosslinked haemoglobins with those of native HbA ($P_{50}=5.0$ mmHg, Hill coefficient=3.0) shows that $\alpha_2\beta^1S^{82}\beta$ binds oxygen with an increased affinity, whereas $\alpha_2\beta^1Tm^{82}\beta$ and $\alpha_2\beta^{1,82}Tm^{82}\beta$ show reduced oxygen affinities; however, all remain cooperative^{8,9}. Crystals of the carbonmonoxide form of $\alpha_2\beta^1S^{82}\beta$, $\alpha_2\beta^1Tm^{82}\beta$ and $\alpha_2\beta^{1,82}Tm^{82}\beta$ were grown at room temperature by the batch method of Perutz¹⁰ in 2.45 M, 2.35 M and 2.35 M sodium-potassium phosphate solutions, respectively, following carbon monoxide saturation. The $\alpha_2\beta^1S^{82}\beta$ crystals are isomorphous with native carbonmonoxide haemoglobin whereas the Tm crosslinked haemoglobins, $\alpha_2\beta^1Tm^{82}\beta$ and $\alpha_2\beta^{1,82}Tm^{82}\beta$, are not. Data for the $\alpha_2\beta^1S^{82}\beta$ and $\alpha_2\beta^{1,82}Tm^{82}\beta$ haemoglobins were collected at room temperature with an Area Detector Systems Corporation (ADSC) area detector using a Rigaku RU200-H rotating anode generator for the X-ray source (40 kV, 150 mA) and the data were processed with the software provided by ADSC. Data for the $\alpha_2\beta^1Tm^{82}\beta$ were collected with an R-Axis II imaging plate at Molecular Structure Corporation (The Woodlands, Texas) and processed with software provided by MSC. The native carbonmonoxide haemoglobin structure minus water molecules was used as the starting model for the $\alpha_2\beta^1S^{82}\beta$ structure and the crosslinker was located in a difference Fourier map and fitted using FRODO²⁵. The model was refined using TNT¹¹. The $\alpha_2\beta^1Tm^{82}\beta$ haemoglobin was solved by Molecular Replacement using the MERLOT¹² software package. The native carbonmonoxide $\alpha\beta$ dimer⁷, minus water, was used as the starting model. Two large peaks in both the rotation and translation functions located the molecule and confirmed the presence of a tetramer in the asymmetric unit (ASU). Refinement¹¹ and difference Fourier maps located the trimesoyl crosslinker, which was fitted using FRODO²⁵. Subsequent cycles of refinement followed until convergence. This structure, minus the crosslinker and waters, was used as the starting model for the $\alpha_2\beta^{1,82}Tm^{82}\beta$ model. The crosslinker was located in a difference Fourier map and the structure was refined to convergence. Coordinates are being deposited in the Brookhaven Protein Data Bank and are available from R.G.B. Full details of the structures will be reported elsewhere (M.A.S. et al., manuscript in preparation). $R_{\text{sym}} = \sum |I_o - \langle I \rangle| / I_o$, where I_o is the observed intensity, and $\langle I \rangle$ is the average intensity from multiple observations of symmetry related reflections. $R\text{-factor} = \sum ||F_o| - |F_c|| / \sum |F_o|$.

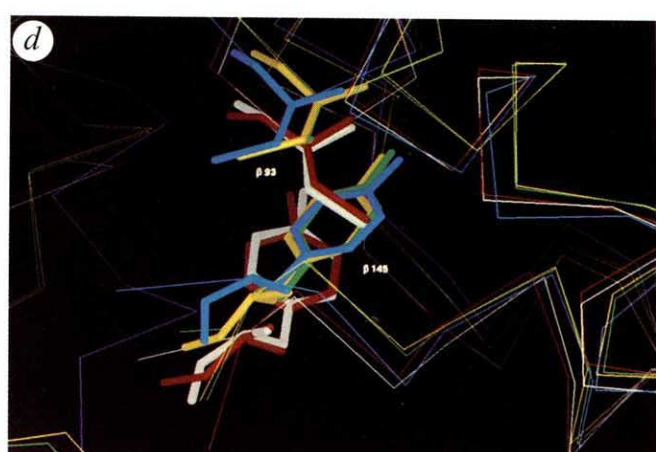
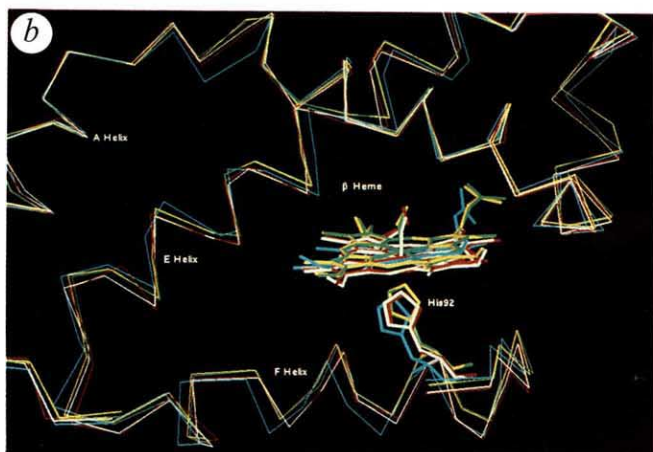
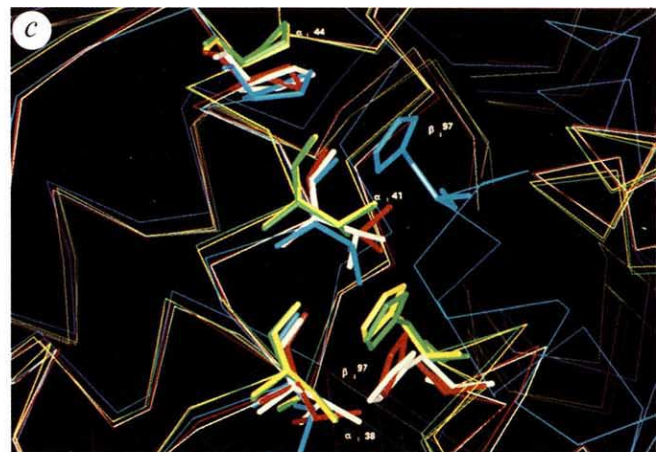
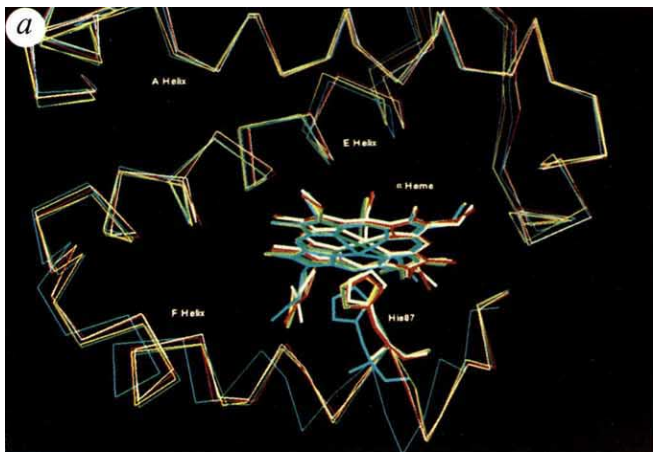
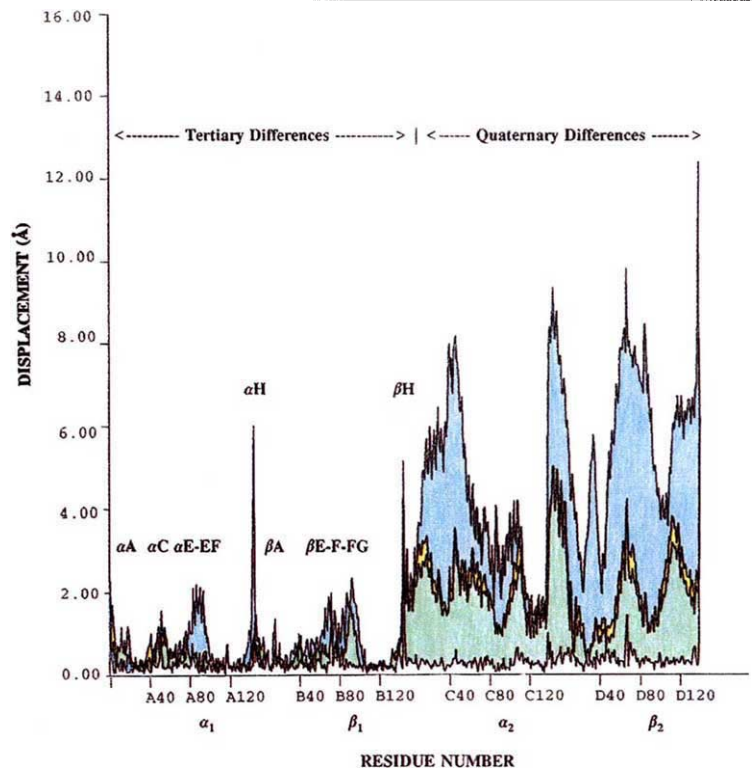
resolution (Table 1). This crosslinked haemoglobin served as the starting model, minus crosslinker, for refinement of the triply linked $\alpha_2\beta^{1,82}Tm^{82}\beta$, which converged to a final R -factor of 13.5% at 1.8 Å resolution (Table 1). The final $2F_o - F_c$ electron density map corresponding to the crosslinker region of $\alpha_2\beta^{1,82}Tm^{82}\beta$ is shown in Fig. 1. To our knowledge, the structure of $\alpha_2\beta^{1,82}Tm^{82}\beta$ provides the first example of a rationally designed, triply linked protein⁹.

Upon binding carbon monoxide, the $\alpha_1\beta_1$ dimer of HbA undergoes a 13.6° rigid body rotation with respect to the $\alpha_2\beta_2$ dimer¹. This rotation is also observed for $\alpha_2\beta^1S^{82}\beta$ (Fig. 2). However, the $\alpha\beta$ dimers of $\alpha_2\beta^1Tm^{82}\beta$ and $\alpha_2\beta^{1,82}Tm^{82}\beta$ (TmHbs) are restricted by the trimesoyl group and rotate only 7.4° and 8.5°, respectively, suggesting that they are trapped in a

transitional conformation (Fig. 2). The tertiary and quaternary differences between the crosslinked haemoglobins and deoxy- and CO-HbA were analysed by a series of α -carbon coordinate-difference plots (Fig. 3). These plots confirm the identical conformations of $\alpha_2\beta^1S^{82}\beta$ and CO-HbA, and demonstrate that simply crosslinking the α -NH₂ of β Val1 of one $\alpha\beta$ dimer to the ϵ -NH₂ of β Lys82 of the other $\alpha\beta$ dimer does not induce large structural perturbations. Most important, these plots reveal that, unlike the structures of other proposed transitional intermediates such as R2-HbA¹³, YpsilantiHb¹⁴ and CN-metHb¹⁵, those of the TmHbs lie directly on the T-to-R conformational pathway.

The most significant structural differences between the TmHbs and CO-HbA and deoxy-HbA are found in elements involved

FIG. 3 Coordinate-difference plot (CDP) showing the differences between corresponding α positions of deoxyhaemoglobin (background, shaded in blue), $\alpha_2\beta^1\text{Tm}^{82}\beta$ (near background, shaded in yellow), $\alpha_2\beta^1\text{Tm}^{82}\beta$ (foreground, shaded in green) and $\alpha_2\beta^1\text{S}^{82}\beta$ (white) after their $\alpha_1\beta_1$ interfaces are overlaid on CO-HbA as in Fig. 2. The ordinate indicates the displacement in Å for the corresponding α atoms and the abscissa indicates the residue number where the A and B chains correspond to the α_1 and β_1 chains, respectively, and the C and D chains, to the α_2 and β_2 chains, respectively. The region designated 'tertiary changes' corresponds to the $\alpha_1\beta_1$ region which was overlaid and refers to structural alterations between tertiary elements, for example, helical displacements. The structural elements that undergo the most significant displacements are labelled on the plot. The region labelled 'quaternary changes' corresponds to the $\alpha_2\beta_2$ region, which was not overlaid, and corresponds to quaternary differences between deoxyhaemoglobin, $\alpha_2\beta^1\text{Tm}^{82}\beta$, $\alpha_2\beta^1\text{Tm}^{82}\beta$ and $\alpha_2\beta^1\text{S}^{82}\beta$ and CO-HbA. The quaternary differences between the TmHbs and CO-HbA are made obvious by these plots and suggest that the TmHbs assume an intermediate quaternary conformation. A similar overlay of the $\alpha_2\beta_2$ interfaces of deoxyhaemoglobin, $\alpha_2\beta^1\text{Tm}^{82}\beta$, $\alpha_2\beta^1\text{Tm}^{82}\beta$ and $\alpha_2\beta^1\text{S}^{82}\beta$ onto that of CO-HbA produces a nearly identical CDP.



directly in the T-to-R transition, namely, the A, C, E and H helices and EF turns of the α -subunits and the A, E, F and H helices and FG turns of the β -subunits (Fig. 3). Inspection of these structural elements reveals that relaxation to the R conformation is incomplete or has not started, and that their locations result in unfavourable steric pressure or strain, which is the probable cause of the reduced oxygen affinities of both subunits of the TmHbs. Specifically, their α -subunit E helices are essentially in the T-state position (Fig. 4a), whereas the E helices of the β -subunits are located between the T and R positions (Fig. 4b). Ultraviolet Raman studies have shown that movement of the E helices of haemoglobin is critical to relieve the strain imposed by ligand binding¹⁶. Also intermediate are the locations of the F helices of the β -subunits, which have moved only 0.7 Å in $\alpha_2\beta^1\text{Tm}^{82}\beta$ and 0.6 Å in $\alpha_2\beta^{1,82}\text{Tm}^{82}\beta$ from the T-state position, as compared with a 1.4 Å shift in CO-HbA (Fig. 4b). Their fully ligated haems have also not reached the R-state location, but there is no haem doming. To maintain proper coordination to the haem Fe^{2+} , the proximal histidine, βHis92 (F8), which retains its T-state χ^1 torsion angle, is significantly displaced towards the haem, implying a parabolic trajectory of F8 in the T-to-R transition (Fig. 4b). These results suggest that the move-

ments of the β -subunit E and F helices and haems are coordinated.

In contrast to the β -subunit F helices, the F helices of the α -subunits have reached the R-state location in the TmHbs (Fig. 4a). The α -subunit haems, which are also fully ligated and planar, are in the T-state location, however, implying that they move independently of their F helices. The conformational transition from the T-state E helices to the R-state F helices is accommodated by the EF turns, which are structurally intermediate (Fig. 4a). The α -subunit proximal histidine, αHis87 (F8), is also positioned between its T- and R-state locations to coordinate the haem Fe^{2+} properly. Thus, the T-to-R state transitional pathways of the α and β haems are different. This finding is in accord with the structures of the T-state liganded haemoglobins, T(α -oxy, β -deoxy)Hb and T(α -met, β -met)Hb^{17,18} and probably reflects the different packing and chemical environments of the α (tight) and β (looser) haems.

Other notable features of these intermediates are found in the switch region and the β -subunit carboxy termini of the TmHbs. Inspection of the switch reveals that, although located in its R-state position between $\alpha_1\text{Thr38}$ and $\alpha_1\text{Thr41}$, $\beta_2\text{His97}$ is displaced significantly towards its T-state location (Fig. 4c). At the C termini, all critical T-state salt bridges, including that between βHis146 and βAsp94 , are disrupted⁷, yet the side chains of their neighbouring residues, βTyr145 and the reactive βCys93 (refs 19,20), maintain their T-state locations, which suggests that their movements occur late in the T-to-R transition (Fig. 4d).

In conclusion, the carbonmonoxy structures of $\alpha_2\beta^1\text{Tm}^{82}\beta$ and $\alpha_2\beta^{1,82}\text{Tm}^{82}\beta$ are probable snapshots of haemoglobin's nascent R state and can explain their lowered oxygen affinities. Using these crosslinked haemoglobins, this idea could also be investigated using dynamic techniques such as ultraviolet Raman^{16,21,22} and ultrafast near-infrared^{23,24} spectroscopy. Our results, combined with the finding of an inverse correlation between the O_2 affinity and crosslinker bridging distance in a series of $\beta^1\text{X}^{82}\beta$ crosslinked haemoglobins⁸, suggests that the structures of additional haemoglobin intermediates, either more or less R-like, can be trapped by using crosslinkers of different lengths, an idea that might be extended to other multistate proteins. □

◀ FIG. 4 Snapshots of the T-to-R transition in haemoglobin. All views are the result of the $\alpha_1\beta_1$ overlay described in Fig. 2 legend. As in Fig. 1, deoxyhaemoglobin is shown in blue, $\alpha_2\beta^1\text{Tm}^{82}\beta$ in yellow, $\alpha_2\beta^{1,82}\text{Tm}^{82}\beta$ in green, $\alpha_2\beta^1\text{S}^{82}\beta$ in white and CO-HbA in red. a, The α_1 haem environments showing the proximal histidines, αHis87 (F8) and the haem groups as solid rendered bonds and the A, E and F helices as Ca traces. The T-state positions of the E helices and haems of the TmHbs are evident, as are the R-state positions of their F helices. The A helices of the TmHbs are also closer to the T-state position, a finding that is consistent with recent UV-Raman studies¹⁶. The α haems are fully ligated and planar. The T-state positions of the E helices and the α haems and the R-state positions of the F helices result in steric pressure which requires F(8) to move to a position between its normal T and R state locations so that the Fe^{2+} atom of the α haem maintains proper octahedral geometry. b, The β_1 haem environments showing the proximal histidines, βHis92 (F8) and the haem groups as solid-rendered bonds, and the A, E and F helices as Ca traces. The intermediate locations of the A, E and F helices of the TmHbs are evident, as are the significant displacements of their proximal histidines towards the haem. Despite the strain resulting from the intermediate nature of the E and F helices of the TmHbs, the β haems are fully ligated and planar. The shifts of the β F helices were quantified (see text) by averaging the Ca displacements (Å) of each F helix residue of deoxyhaemoglobin, $\alpha_2\beta^1\text{Tm}^{82}\beta$, $\alpha_2\beta^{1,82}\text{Tm}^{82}\beta$ and $\alpha_2\beta^1\text{S}^{82}\beta$ relative to the corresponding residues in CO-HbA. c, The switch region of haemoglobin, which is composed of residues 97–102 from the β_2 FG corner and G helix and residues 38–44 from the C helix and CD corner of the α_1 subunit, demonstrating the significant displacements of the key switch residue $\beta_2\text{His97}$ of $\alpha_2\beta^1\text{Tm}^{82}\beta$ (yellow) and $\alpha_2\beta^{1,82}\text{Tm}^{82}\beta$ (green) towards the T-state position. The distances from the $\beta_2\text{His97}$ Ca atoms of $\alpha_2\beta^1\text{Tm}^{82}\beta$ and $\alpha_2\beta^{1,82}\text{Tm}^{82}\beta$ to the corresponding $\beta_2\text{His97}$ Ca atom in CO-HbA (red) are 1.26 and 1.47 Å, respectively, compared to a distance of 7.12 Å for deoxyhaemoglobin (blue). Despite these displacements, the $\beta_2\text{His97}$ of the TmHbs are clearly in the R conformation, as evidenced by their locations between $\alpha_1\text{Thr38}$ and $\alpha_1\text{Thr41}$. In deoxyhaemoglobin, His 97 sits between $\alpha_1\text{Thr41}$ and $\alpha_1\text{Pro44}$. d, The pocket formed by the β -subunit F and H helices showing the T-state position of the side chains of βCys93 and βTyr145 of $\alpha_2\beta^1\text{Tm}^{82}\beta$ (yellow) and $\alpha_1\beta^{1,82}\text{Tm}^{82}\beta$ (green). The side chains of βCys93 and βTyr145 of the $\alpha_2\beta^1\text{S}^{82}\beta$ haemoglobin (white), however, are clearly in the R-state location. Deoxyhaemoglobin and CO-HbA are shown in blue and red, respectively.

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***Australopithecus ramidus*, a new species of early hominid from Aramis, Ethiopia**

Tim D. White, Gen Suwa & Berhane Asfaw

Nature **371**, 306–312 (1994)

IN this article we attributed hominid fossils from Aramis to *Australopithecus ramidus* sp. nov. We noted that generic placement was conservative and that *ramidus* might represent a new genus. Meanwhile, as *ramidus* is likely to be the sister taxon of the remaining hominid clade, generic separation from *Australopithecus* is appropriate. We hereby make available a new generic nomen for the Aramis fossils described in our article.

Order Primates Linnaeus 1758
Suborder Anthropoidea Mivart 1864
Superfamily Hominoidea Gray 1825

Ardipithecus gen. nov.

Etymology. 'Ardi' means 'ground or floor' in the Afar language. **Type species.** *Australopithecus ramidus* White, Suwa and Asfaw, 1994.

Locality, horizon and associations. These are the same as for *Australopithecus ramidus*.

Diagnosis. Compared with *Australopithecus*, *Ardipithecus* is characterized by less postcanine megadontia, with upper and lower canines larger relative to the postcanine teeth; lower first deciduous molar narrow and obliquely elongate, with large protoconid, small and distally placed metaconid, no anterior fovea, and small, low talonid with minimal cusplike development; temporomandibular joint without definable articular eminence, absolutely and relatively thinner canine and molar enamel; lower third premolar more strongly asymmetrical, with dominant, tall buccal cusp and steep, posterolingually directed transverse crest; upper third premolar more strongly asymmetrical, with relatively larger, taller, more dominant buccal cusp.

Discussion. In late 1994 a mandible of *A. ramidus* was found at Aramis associated with a partial postcranial skeleton, 50 m north of the holotype specimen, and at the same stratigraphic level. Analysis of this specimen has begun, and will provide further features with which to characterize *Ardipithecus*. □

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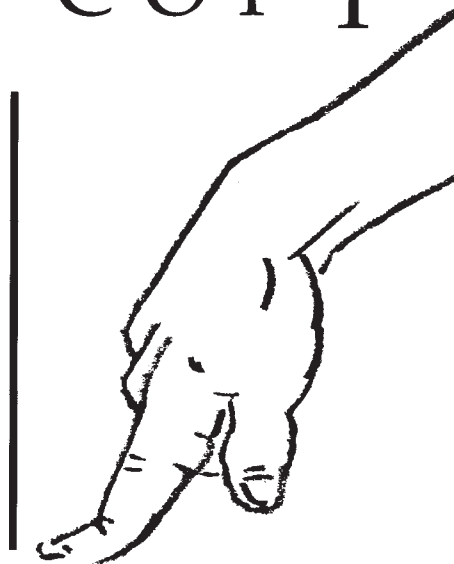
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Nature's sister journals in review

Highlights this month include new approaches to organ transplantation and antitumour vaccines, suspect chromosomes in psychiatric disorders, and insight into the workings of transcription factors of lipases.

Moving a step closer to xenotransplants

LARGELY thanks to improved surgical techniques, solid organ transplantation has become a routine procedure and would go on to become commonplace but for the severe shortage of organs. The possibility of using organs from anatomically appropriate animals in human patients has thus become the Holy Grail of organ transplantation. But the likelihood of this xenotransplantation dream being realized has been thwarted by the violent rejection experienced by organs transplanted across a species barrier. J. Platt and colleagues now describe an ingenious route towards bypassing this block to xenotransplantation on page 423 of this month's *Nature Medicine*.

The all-out rejection of xenografts is mediated by naturally occurring host antibodies that bind to donor endothelial cells and trigger the powerfully destructive complement cascade. Under normal circumstances, complement is held in check by regulatory proteins such as decay accelerating factor (DAF or CD55) and CD59 (a membrane-bound inhibitor of reactive lysis). Transient inhibition of complement activity is possible (using cobra venom factor, for example), but such inhibitors do not seem to be very effective against the heterologous complement of the recipient, so xenografts are particularly prone to rapid rejection.

Platt *et al.* have engineered transgenic swine to express human complement regulatory proteins and have transplanted hearts from these animals into baboons. The pig-human transgenic hearts were grafted onto the necks of the baboons, creating a beating graft, and were monitored until graft rejection, when beating stopped. Three transgenic hearts survived for 4, 11 and 30 hours, an improvement over non-

transgenic hearts, which typically survive for 60–90 minutes. Furthermore, the level of graft damage (interstitial haemorrhage, thrombosis and myocyte injury) was considerably less in the failed transgenic grafts, suggesting that substantially increased survival may be near to hand.

Although the results do not immediately open the way for clinical xenotransplantation, they are an encouraging step towards it.

With further expression of the appropriate transgenic complement regulatory proteins and perhaps reduced expression of the most antigenic of the swine endothelial cell surface proteins, this little piggy may yet go to surgery.

Antitumour vaccines

As tumour cells can express foreign antigens on their surface, they would be expected to elicit immune responses. But the response is usually poor, perhaps because the antigen is only weakly immunogenic or because of an unusual tolerance of the antigen and the accompanying tumour-specific cellular response. On page 471, Z.-K. Pan *et al.* describe a novel strategy for successful immunization against a model tumour.

Listeria monocytogenes is a Gram-positive bacterium with particularly good cre-

dentials as a vaccine vector. Whereas most bacteria are confined to the vacuole in which they enter a cell, *L. monocytogenes* can, by virtue of a virulence factor, disrupt the vacuole membrane and enter the cytoplasm. Once there, bacterial antigen can target the host's MHC class I and class II pathways, inducing a strong cellular immune reaction.

Pan *et al.* used two tumour cell lines (from a murine colon tumour, CT26, and a spontaneous renal cell carcinoma) to express an influenza nucleoprotein. They then tested the ability of a recombinant *L. monocytogenes*, engineered to express and secrete the flu nucleoprotein antigen, to elicit an antitumour response. Mice were immunized with the recombinant *L. monocytogenes* and challenged with large doses of the cancer cells. All ten animals receiving renal carcinoma cells remained tumour-free, so were six of the ten mice that received CT26 cells, and the remaining four had very small tumours compared with those of non-immunized control mice. Mice with established macroscopic tumours were also treated with the vaccine and, in an unprecedented demonstration of the potential of antitumour vaccination, long-lasting regression was noted.

Should this approach prove as successful in a real tumour-antigen system (as opposed to the model system presented here), the way will have been cleared for the first oral anticancer immunotherapy, because *L. monocytogenes*, when administered orally, can induce cellular immune responses in the periphery. □

Schizophrenia chromosome revisited

THAT a group of errant genes plays a part in determining the susceptibility of large numbers of people to developing psychiatric disorders is not in doubt, but unravelling the precise number and identity of the loci responsible has perplexed many distinguished geneticists for years. A false lead was published in *Nature* some six years ago, when Gurling and colleagues presented evidence for a predisposing gene for schizophrenia on chromosome 5, but this and other reports of linkage assignments for complex psychiatric traits, including bipolar affective disorder, could not be confirmed.

Some strongly suggestive evidence in favour of a schizophrenia locus on the short arm of chromosome 6 is now offered on page 41 of the May issue of *Nature Genetics* by S. Diehl and colleagues. They have examined a large sample of 186 Irish families which included more than 500 clinically defined cases of schizophrenia and related disorders. Members of the families were typed with 35 DNA markers, representing about 10% of the genome. Following up a hint of linkage with the factor 13A gene (*F13A1*) near the tip of the short arm of chromosome 6, Diehl's group looked at six other markers on the centromeric side

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genetics

Nature Medicine: other points

- Selenium-deficient diet selects for virulent mutant virus that give rise to heart disease.
- A placental clock controls the length of human pregnancy.
- Response to a lymphocyte toxin suggests that psoriasis is a disease primarily of the immune system, not keratinocytes.
- Non-viral approaches to cancer gene therapy.

of *F13A1*. At least one of these markers, *D6S260*, gave a much higher logarithm of the odds (lod) score of +3.5. Furthermore, in a multipoint analysis using *F13A1* and *D6S260*, the lod score reached 3.9.

The investigators next performed non-parametric linkage analysis, which does not require specification of the mode of inheritance, and thus offers advantages when it comes to studying complex diseases. Analysis of the alleles of DNA markers shared among pairs of affected siblings, and the distribution observed compared with that expected by chance, also gave significant results at *D6S260* and *D6S285*.

These findings suggest that there may well be a schizophrenia gene that maps to chromosome 6p22-pter but, despite the persuasive statistical evidence, confirmation will be required. The authors argue

that their clinical material, both in terms of sheer size and uniform methods of diagnosis, offers distinct advantages over earlier genetic linkage studies. There is also reason to be optimistic about the results: the authors' calculated lod score corresponds to a genome-wide significance level of 2.5%, meaning, they say, that there is only about a 1 in 40 chance that these results might have occurred by chance during a genome-wide search. That may be about as good as can initially be hoped for in searching for genes responsible for complex diseases. It is still too early to say whether the putative locus on chromosome 6 plays a minor role in most of the families with schizophrenia, or a major one in just a few of them.

Regardless of the ultimate fate of the schizophrenia linkage, progress is being made in charting the genes involved in an important form of epilepsy known as 'partial' epilepsy. For many years, the hereditary component of partial epilepsies, so-called because the seizures originate in localized regions of the cerebral cortex, has been in doubt, despite evidence for a hereditary basis for 'generalized' epilepsy. Fresh support comes from two groups (R. Ottman *et al.* on page 56 and H. A. Phillips *et al.* on page 117) who have mapped two distinct loci in two different families with slightly different features to chromosomes 10 and 20, respectively. □

Nature Genetics: other points

- Identification of the gene for X-linked ocular albinism by positional cloning.
- Methylation of CpNpG sequences in mammalian cells.
- The effects of translating CAG repeats in proteins associated with neurodegenerative disorders such as Huntington's disease.
- The association of a missense mutation in lipoprotein lipase with reduced HDL cholesterol levels in premature atherosclerosis.

Cementing interactions

RECOGNITION of individual genes in the vast sea of the genome is a vital step in decoding the genetic blueprint of organisms. Each gene, or class of genes, is tagged by specific promoter sequences which are recognized by specific transcription factors. Although the interaction between transcription factors and their DNA targets is well understood, little is known about how these important proteins avoid the multitude of closely related but irrelevant sequences in the genome. In an effort to understand the mechanism of target discrimination, D. Gewirth and P. Sigler describe on page 386 of this month's *Nature Structural Biology* how they have captured a hybrid member of the steroid receptor family bound to the 'wrong' DNA target.

Nature Structural Biology: other points

- Characterization of a protein folding-defect shows that delayed folding leads to emphysema.
- Building zinc binding into streptococcal protein G.
- A small number of residues are responsible for the stability of thermolysin.
- Structure of a DNA tetraplex demonstrates that adenine bases can also participate in this motif.

The glucocorticoid (GR) and oestrogen (ER) receptors recognize the edges of the bases in the major groove of their DNA target sites primarily through a 'recognition' helix. Gewirth and Sigler have studied the interaction of an oestrogen receptor-like DNA-binding domain (E/TR_{GR}) bound to a glucocorticoid response element (GRE), rather than to its cognate oestrogen response element (ERE). Comparison of this non-cognate complex with the cognate complexes (GR/GRE, ER/ERE) reveals that the extent of the buried surface area (a good indicator of the strength of an interaction) is roughly similar in all cases, even though the non-cognate interaction is considerably weaker.

The presence of a sizeable water-filled cavity in the non-cognate complex (not seen in either of the cognate complexes) provides a possible explanation. The ordered water molecules act a 'caulking' agent to hold the ill-fitting non-cognate complex together, bridging the chemical groups on protein and DNA, but they are also likely to place a heavy entropic burden on the association. So the decreased affinity of the E/TR_{GR} for its non-cognate site may in fact be an unavoidable penalty for

using the fixed water molecules as a mortar to fill the gaps between protein and DNA.

Lipid bilayers and lipolysis

Two other papers tackle the question of how the enzymes that digest lipids cope with the lipids' laminar state — but from opposite directions. One (from B. van den Berg *et al.* on page 402) focuses on structural changes in phospholipase A₂; the second (by G. H. Peters *et al.* on page 395) studies the lipid layer itself.

From the early years of this century it has been known that lipolytic enzymes have a higher activity when presented with their lipid substrates as multimolecular aggregates rather than as individually dispersed molecules. The basis for this phenomenon, termed 'interfacial activation', has fuelled much debate. One camp contests that the enzymes themselves must undergo a conformational change when interacting with lipid layers. A second camp claim that the explanation for interfacial activation lies in the properties of the substrate (for example, a large concentration gradient at the interface).

Peters *et al.* have pursued the substrate route. Using a variety of techniques, including sophisticated X-ray analysis (grazing incidence X-ray diffraction), they have investigated the structure and dynamics of a diglyceride monolayer as a function of surface pressure. They have identified phase transitions that alter the solvent accessibility of the lipids' head groups. These transitions correlate with changes in lipase activity with surface pressure, showing how substrate surface may affect enzyme activity.

Structures of phospholipases derived by X-ray crystallography have failed to identify any conformational changes on substrate binding, but the crystal environment is somewhat different from the situation *in vivo*. Van den Berg *et al.* reason that NMR, which reveals protein structure in solution, might be more successful. They have identified a

major structural change in phospholipase A₂ in the presence of lipid micelles and a substrate analogue. The amino terminus of the enzyme takes up an ordered α -helical conformation, forming a vital hydrogen-bonding network at the enzyme's active site.

These two papers provide support for both sides of the interfacial activation debate and indicate that its resolution may be more complex than the models of either group of protagonists. It would appear that structural changes in both the enzyme and substrate can influence lipase activity. □

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